Determining the Organisms, Pathways of Infection and Risks for Ovine Mastitis

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Declaration

This thesis is submitted to the University of Warwick in support of my application for the degree of Doctor of Philosophy. It has been composed entirely by myself apart from the coding in Chapter 5 which was completed by Dr Ron Crump. It has not been submitted in any previous application for any degree apart from Chapter 2 where the questionnaire was designed during my research masters and Chapter 5 which has been submitted as a report for the Texel Society.

Summary

The aims of the first study were to estimate the incidence rate of clinical mastitis (IRCM) for suckler ewes (ewes rearing lambs for meat production) in England and to identify potential risk factors. A postal questionnaire was sent to 999 randomly selected English sheep farmers in 2010 to gather data on the IRCM and flock management practices for the calendar year 2009. The mean IRCM per flock was 1.2 / 100 ewes / flock / year (0.0-19.1). The IRCM was 2.0, 0.9 and 1.3 / 100 ewes / year for flocks that were always housed, always outdoors and a combination of both respectively. Six mixed effects over-dispersed Poisson regression models offset by flock size were developed to identify management practices associated with IRCM. Of particular interest were environmental factors, as indicated by the affect flooring and indoor versus outdoor rearing had on the IRCM, and host susceptibility as indicated by the association of IRCM with udder conformation (a potentially heritable trait).

Based on these findings, the role of the environment was first considered by investigating the potential sources of mastitis-causing pathogens. Milk samples and udder skin swabs were taken from 27 pedigree Texel ewes with clinical mastitis and 3 ewes showing no clinical signs in order to identify whether udder skin could act as a bacterial reservoir for intramammary infections. Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS) was used to identify isolates to species level and compared to pulsed-field gel electrophoresis (PFGE) to assess the capabilities of MALDI-ToF-MS as a strain differentiation tool. MALDI-ToF-MS had good correspondence with PFGE for all species tested in this study including *Staphylococcus warneri, Staphylococcus equorum, Rhodococcus corprophilus, Bacillus pumilus* and *Micrococcus luteus.* The same strain type was found in mastitic milk and on ewe udder skin for *Staphylococcus warneri* and *Rhodococcus corprophilus* indicating a possible reservoir of bacteria that might enter the mammary gland.

The third study built upon the findings from the second study by identifying the role of lamb mouths and ewe udder skin as potential transmission pathways of mastitis causing bacteria by sampling these environments and ewe milk over time in a longitudinal study of 21 ewes over 10 weeks. Twenty-six bacterial species were found in more than one location (lamb mouth, ewe udder skin and/or ewe milk), many of which have previously been associated with mastitis. Potential transmission events and persistence of the same bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time were identified for the first time in suckler ewes using MALDI-ToF-MS for a variety of bacterial species.

Having identified udder conformation as the most significant factor associated with IRCM in the first study, the final study aimed to investigate whether udder and teat conformation are heritable traits that affect mastitis. A total of 968 records were collected from 10 Texel flocks over a period of 3 years (2012-2014) from England, Wales and Scotland to assess whether udder and teat conformation were heritable traits associated with higher levels of chronic mastitis. Univariate quantitative genetic parameters were estimated using individual animal and sire models. The heritabilities for teat length and teat placement were greatest (0.42 and 0.35, respectively). The remaining traits (traits that generally describe the volume of the

udder) were of moderate to low heritability. Univariable logistic regression was used to identify the phenotypic association between udder traits and chronic mastitis.

The work in this thesis has addressed several gaps in the knowledge in mastitis epidemiology by providing the first estimate for the IRCM in suckler flocks in England, and generating hypotheses for factors that may affect the IRCM. The role of the ewe's environment and heritable traits that could potentially affect a ewe's likelihood of getting mastitis were subsequently investigated. The results have contributed to our understanding of the factors associated with the development of mastitis.

Abbreviations

% w/v	Percent weight to volume
% v/v	Percent volume to volume
AN	Acetonitrile
ANOVA	Analysis of variance
B	Beta
BHI	Brain heart infusion agar
BSA	Bovine serum albumin
BTS	Bacterial test standard
CCI	Composite correlation indices
CHEF	Contour-clamped homogeneous electric field
CI	Confidence interval
CM	Clinical mastitis
CMT	California mastitis test
CNS	Coagulase-negative staphylococci
DIC	Deviance information criterion
Exp	Exponential
FCM	Flow cytometry measurement
FSD	Flock-by-scoring date factor
GLMM	Generalized linear mixed model
INLA	Bayesian integrated nested laplace approximation
IRCM	Incidence rate of clinical mastitis
MALDI-ToF-MS	Matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MLST	Multi locus sequence typing
OD	Optical density
OR	Odds ratio
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
RR	Risk ratio
SBA	Sheep's blood agar
SCC	Somatic cell count
SCM	Subclinical mastitis
SD	Standard deviation
SDS	Sodium dodecyl sulfate
SE	Standard error
SNPs	Single nucleotide polymorphisms
TE	Tris ethylenediaminetetraacetic acid (edta)
TFA	Trifluoroacetic acid
UPGMA	Unweighted pair group method with arithmetic mean
Var	Variance
WST	Whiteside test

Chapter 1. Literature review

Endemic diseases such as mastitis result in both a direct and indirect economic loss for the industry. In both ewes kept to produce milk (hereafter dairy ewes) and in ewes kept to produce lambs for human consumption (hereafter suckler ewes), costs of diagnosis, treatments, preventive measures, labour, carcass disposal, and ewe replacements result in substantial economic loss (Hogeveen *et al.* 2011, Pinzón-Sánchez *et al.* 2011).

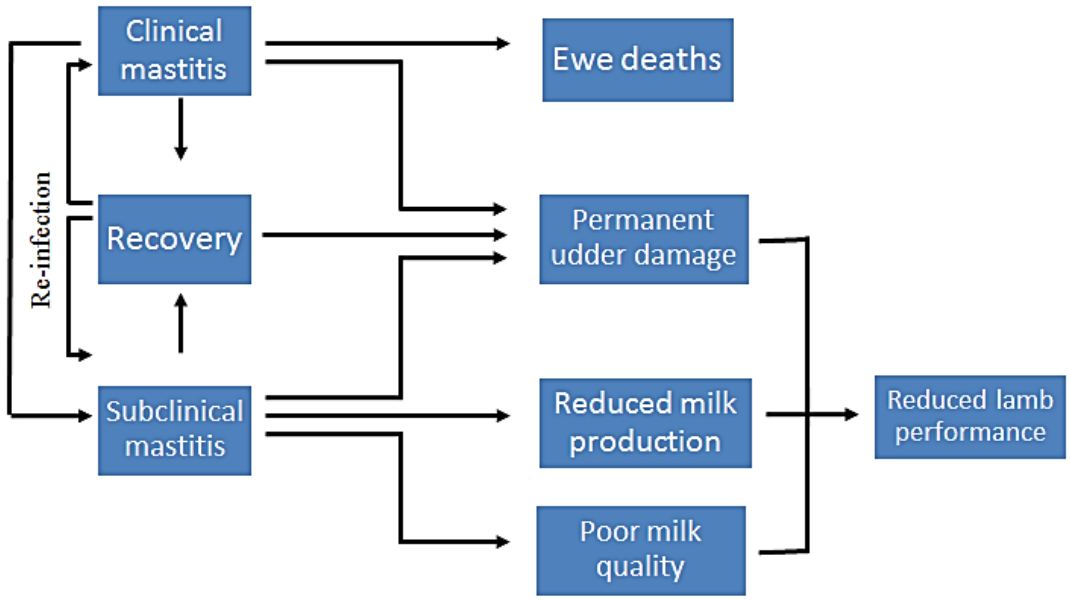
In dairy ewes, decreased milk yield, by up to 55% (Saratsis *et al.* 1999), downgrading of milk due to high somatic cell counts (SCC) and higher bacterial counts contribute to the economic losses attributed to mastitis (Fthenakis & Jones 1990a).

In suckler ewes, costs occur through decreased live-weight gain of lambs, and loss of lambs that would have been reared by the affected ewe. In addition, lamb performance is decreased in lambs whose mothers have subclinical mastitis, due to decreased milk production (Fthenakis & Jones 1990a, Keisler *et al.* 1992), and changes in suckling behaviour (Gougoulis *et al.* 2008a), which is particularly important in suckler flocks. In a recent study of 67 ewes followed through lactation, lower lamb growth rates were associated with an increase in the dams somatic cell count (>400,000 cells/ml) (Huntley *et al.* 2012). Improvement in the management and therefore prevalence of mastitis would benefit the health and welfare of sheep and lambs and help to reduce economic losses.

An accurate estimate for the cost of mastitis to the UK sheep industry is not available, because costing farm animal disease is complex (Conington *et al.* 2008). Using computer models of flock dynamics, however, a 10% decrease in the risk of contracting mastitis (through control methods) has been estimated to be worth £8.40 per ewe in the Texel breed (Conington *et al.* 2008). In cows, the economic losses attributed to mastitis are estimated to be between €61 and €97 per cow-year based on dynamic programming models and herd-simulation models, respectively (Bar *et al.* 2008, Hagnestam-Nielsen & Østergaard 2009). A summary of the economic implications of mastitis are shown in Figure 1.1. There are, however, methods that can reduce the economic effect of mastitis on a flock, for example by supplementing

lamb feed in suckler flocks with mastitis in order to compensate for the reduction in milk yield. This would reduce the effect of mastitis on lamb performance (Keisler *et al.* 1992).

Figure 1.1: Adverse implications of mastitis. Image adapted from (Watson & Buswell 1984).



1.1. Mastitis: an overview

Mastitis is defined as an infection of the mammary gland primarily caused by bacteria that result in inflammation of the mammary gland tissue (Khan & Khan 2006). Mastitis can also be caused by viral infections, trauma, allergies, and physiological and metabolic changes (Bergonier *et al.* 2003). Ewes that have mastitis may experience discomfort and pain in the udder, or be systemically sick or die. Mastitis has typically been categorised as clinical (CM), where an animal has overt clinical signs, or subclinical (SCM), where clinical signs are absent.

Clinical mastitis is typically classified as per-acute, acute or chronic and may be severe or mild. It can result in death, visible swelling of the udder, abnormal discharge from or pain in the udder, milk composition modifications (Firat 1993, Gonzalez *et al.* 1990, Hovinen *et al.* 2008, Klaas *et al.* 2004), lethargy, weight loss and increased rectal temperature (Arsenault *et al.* 2008). Farmers often diagnose acute clinical mastitis in sheep using whole animal signs such as lethargy or malaise or behavioural changes such as lameness, or kicking away sucking lambs. Typically,

clinically affected udder halves do not return to normal function (Mørk *et al.* 2007). Chronic mastitis is often detected when farmers palpate ewes' udders at weaning when deciding whether or not to retain a ewe for breeding.

Subclinical mastitis can lead to a reduction in milk yield and altered milk composition (Bergonier & Berthelot 2003, Conington *et al.* 2008, Fthenakis & Jones 1990a). In sheep, there is no universally accepted definition of subclinical mastitis with no established 'normal' somatic cell count and as such different studies may define cases differently.

Clinical and subclinical mastitis are fairly rigid classifications of disease, and it can be argued that there is a single udder disease in sheep, caused by a variety of bacteria, with different clinical manifestations. These categorisations depend very much on the degree of inspection given to an animal. In suckler ewes it is quite likely that changes in the milk alone are not detected by farmers and so these fall into the category of subclinical infection. In milk sheep and dairy cows such changes would be detected because the udder and milk are inspected twice each day.

It is thus important to consider mastitis as a spectrum from infection to disease that shifts the status of the udder from healthy to diseased. To date, much of the research on mastitis has tended to focus on causative species and clinical signs, due to technical limitations in strain differentiation methods. Therefore we do not have a clear understanding of the dynamics of the disease, specifically how and when ewe's udders become infected with pathogenic strains.

1.1.1. Detection of mastitis

The ability to detect cases, to identify accurately the causative bacteria to a suitable level, enables rapid intervention and the use of appropriate treatment (Ghebremedhin *et al.* 2008). Key methods of detection include visual observation of the ewe and udder, bacterial culture of milk samples, the California Mastitis Test (CMT), the Whiteside Test (WST), flow cytometry (FCM), and measurement of somatic cell count (SCC).

Traditionally bacterial culture has been used to identify the causative agents of mastitis. Identification is normally based initially on fast and simple tests such as

Gram staining, catalase and oxidase tests, with secondary phenotypic tests used to complete the identification (Carbonnelle *et al.* 2011, Sintchenko *et al.* 2007). Bacterial culture was considered fast, accurate, and low cost for many decades; the gold standard for the identification of bacteria to at least genus if not species level (Ghorbanpoor *et al.* 2007). In recent years, ongoing revolution in bacteriology by means of molecular rather than phenotypic methods have deemed these tests labour intensive, slow, imprecise on occasion, and expensive (Carbonnelle *et al.* 2011, Ghorbanpoor *et al.* 2007).

Somatic cell count is the number of somatic cells present per millilitre of milk (cells/ml) (Lafi 2006). Somatic cells are comprised of neutrophils, macrophages and ewe epithelial cells (Oviedo-Boyso *et al.* 2007). When the udder half is infected with bacteria, inflammation is triggered and the number of neutrophils recruited to the udder rises rapidly. This increases the somatic cell count which can be counted by Coulter or Foss counters and is used to detect cases of subclinical or clinical mastitis in dairy cows. The somatic cell count gives an indication of whether an immune response has begun and when measured at regular intervals can indicate the timing and extent of this immune response and has been used in cows and sheep (González-RodríGuez *et al.* 1995, Rupp & Boichard 2000).

It should be noted that the somatic cell count is influenced by other factors aside from infection stage and pathogen such as physiological status of the animal, lactation stage, and milk yield. The distributions of somatic cell count between ewes with different udder health infection statuses overlap and therefore is it difficult to define a somatic cell count threshold for mastitis (Carta *et al.* 2009). However, a somatic cell count of >200,000 cells/ml is considered to be a rational threshold for the presence of bacterial infection in cows based on research on somatic cell counts of cows with and without mastitis (Dohoo & Meek 1982, Green *et al.* 2006, Madouasse *et al.* 2010). There is as yet no accepted somatic cell count threshold for the detection of infection in the udder of sheep. Whilst there is little regulation of the somatic cell count in sheep milk produced commercially, the EU legal limit for raw goat or sheep milk intended for manufacture of dairy produce is 500 x 10^3 cells/ml (European Commission 1992).

Although an increased somatic cell count can be used as a proxy for bacterial infection, there is some debate over how low somatic cell counts can affect an animal's susceptibility to mastitis. Research has indicated a non-linear relationship between somatic cell count and the odds for clinical mastitis in cows (Peeler *et al.* 2003). The importance of somatic cells in the protection of the udder against clinical mastitis has become apparent (Peeler *et al.* 2003) with low herd bulk milk somatic cell counts resulting in cows being at an increased risk of toxic mastitis (Green *et al.* 1996). Somatic cell count also varies depending on the bacterial species isolated from the infected udder half which makes using somatic cell count as a linear indicator of bacterial infection in the udder complicated (González-RodríGuez *et al.* 1995, Peeler *et al.* 2003).

Ideally a combination of detection methods, for example somatic cell count and bacterial identification should be used in order to diagnose and effectively treat the infection.

1.1.2. Treatment and prevention of mastitis

Early detection of mastitis is necessary to improve treatment success. If detection and therefore treatment is too late, it may not save the affected udder half or the ewe. Ewes with mastitis are usually treated or culled. Treatment for clinical mastitis is often an intramuscular antibiotic, although it is not used by all farmers due to the expense. Animals may also be re-infected after treatment and recovery. In order for antibiotics to be effective, information about the aetiology, history of clinical or subclinical mastitis cases and parity are useful, but are not always available (Pinzón-Sánchez & Ruegg 2011). In addition, antibiotics are occasionally ineffective in controlling infections caused by certain bacteria resulting in reservoirs of bacteria within a herd or flock that results in chronic and/or persistent infections (Pellegrino *et al.* 2010).

Prevention of mastitis altogether is unlikely; it would be unrealistic to expect a single prevention protocol to be effective for all mastitis cases, due to the multifactorial nature of the disease and the numerous causative agents. However, advancing our understanding of mastitis, including identifying risk factors for mastitis, the methods by which causative agents gain access to the udder and the pathway of infection may

allow improvements in management practices, or preventative measures that can be used to reduce the levels of infection in a flock.

Control strategies target sources and transmission. In order to control sources, existing infection must be removed through the culling of ewes with clinical mastitis, or through intramammary antibiotics during the dry period (dry-ewe therapy) for ewes with subclinical mastitis (Bergonier & Berthelot 2003, Gonzalo *et al.* 2005, Kiossis *et al.* 2007).

Effective management practices can also be used to control disease transmission. Milking machine standards should be met; regular maintenance of milking machinery to reduce traumatic damage to the udder must be implemented. Gonzalo *et al.*, (2005) found that udder health (by proxy of bulk tank somatic cell count) could be improved by optimising milking machine standards, for example by having reduced vacuum levels and elevated pulsation rate during machine milking (Gonzalo *et al.* 2005). Milking machinery and/or milkers' hands should also be cleaned thoroughly to reduce the likelihood of spread of contagious bacteria. Management practices such as teat dipping are regularly used in order to prevent new intramammary infections (Fox 1992, Neave *et al.* 1969, Philpot & Pankey Jr 1975). These management practices generally relate to dairy animals; there is a lack of information on effective management practices to control disease transmission in suckler ewes.

In line with finding preventive measures to control mastitis, breeding for resistance is a long-term strategy that may reduce mastitis prevalence in sheep. Despite the accumulation of research on the genetic element of mastitis in cattle, little has been carried out on heritable traits that are associated with mastitis in sheep and even these focus on dairy rather than suckler ewes. This has resulted in a tendency to select towards traits that are profitable for dairy ewes if any, such as milk yield, rather than away from mastitis.

Selecting for increased genetic resistance to mastitis tends to be indirectly through the prediction of health status of the udder based on inflammatory factors such as somatic cell count. Utilising direct selection- relating to the diagnosis of infection such as bacteriological examination and or observation of clinical cases is unusual in

genetics studies due to the large amount of records required for robust modelling. However, a genetic correlation between somatic cell count and udder infection status (using bacteriological analyses) was found (0.93) (Tolone *et al.* 2013). This would indicate that a reduction in somatic cell count could result in a reduction in mastitis incidence. This has been shown in a study of 2 lines of Lacaune ewes divergently selected for somatic cell count which showed that selecting for a reduction in somatic cell count lead to increased resilience to intramammary infections. This included lower incidence of clinical mastitis, prevalence of chronic (as indicated by mammary abscesses) and subclinical intramammary infections, and a better ability to recover from infections (Rupp *et al.* 2009). However it must be noted that selecting for low somatic cell count could damage the ability of the animal to initiate an appropriate immune response to pathogens and therefore resist infection.

Using repeatability test-day models, somatic cell count heritability estimates have ranged from 0.04 to 0.16 (Baro *et al.* 1994, El-Saied *et al.* 1998, Hamann *et al.* 2004, Othmane *et al.* 2002, Riggio *et al.* 2007, Serrano *et al.* 2003) and 0.11 to 0.18 for the average somatic cell count during lactation (Barillet *et al.* 2008, Barillet *et al.* 2001, Legarra & Ugarte 2005, Mavrogenis *et al.* 1999, Rupp *et al.* 2003).

In parallel with cattle, the association between genetic and phenotypic factors (specifically udder conformation) and mastitis in dairy ewes (Casu *et al.* 2010, Legarra & Ugarte 2005, Marie-Etancelin *et al.* 2005) has been shown. In a study of 7558 records from 2262 primiparous ewes, the somatic cell count increased as cistern height increased and degree of separation and udder depth decreased. The genetic correlations between somatic cell count and udder depth, teat placement and degree of suspension were favourable (0.39-0.50) apart from for degree of separation (0.05). The heritability estimates for udder traits ranged from 0.32 to 0.50 (Casu *et al.* 2010). In a study of 82019 primiparous ewes from 352 flocks, the genetic correlations among udder traits were moderate to high (0.14-0.49) and between the estimated breeding values (EBVs) for somatic cell count and udder-type traits were weak but favourable (0.1-0.2). The heritability estimates were slightly lower (0.19-0.33) (Marie-Etancelin *et al.* 2005) than those found by Casu *et al.*, 2010.

The same pattern was found in a study of Laxta sheep, whereby the genetic correlations among udder traits were moderate for all traits (0.34-0.58) apart from

teat size (0.05-0.31). Genetic correlations between somatic cell counts ranged from 0.01 for teat placement to 0.29 for teat size. The heritability estimates ranged from 0.26 to 0.40 (Legarra & Ugarte 2005).

The effect of udder conformation traits on somatic cell count has been shown in a recent study cohort study of 67 suckler ewes, where pendulous udders and greater cross-sectional area of the teats were associated with an increase in somatic cell count (Huntley *et al.* 2012).

Previous studies have indicated some udder conformation traits (in particular teat placement) have high repeatability within (de la Fuente *et al.* 2011, Fernández *et al.* 1995) and across lactation (de la Fuente *et al.* 2011) whilst others relating to udder size and therefore milk yield were affected by lactation and flock (Serrano *et al.* 2002). These traits also had lower heritability (Serrano *et al.* 2002).

These traits are likely to be associated with mastitis due to affecting milk production, milk let down and suckling ease (Huntley *et al.* 2012). Based on the studies discussed, selection based on udder morphology traits could potentially result in improved resilience against mastitis.

1.2. Epidemiology of mastitis

Several studies have identified a range of clinical and subclinical incidence and prevalence rates across the world (Table 1.1). The only estimate for the incidence of clinical mastitis in suckler ewes was 1.2% in Canada, whereas the prevalence of subclinical mastitis was between 9.2-50.0%. The only estimate for the prevalence of subclinical mastitis in suckler ewes in England is 11.7%. There is no incidence rate for clinical mastitis for suckler ewes in England.

Table 1.1: Worldwide incidence and prevalence rates of clinical and subclinical mastitis in flocks of sheep.

Location	Flock	Size of study	Mastitis classification	Prevalence	Incidence	Reference
Wales	Suckler	8 flocks, 1093 ewes	Subclinical	14.0%		(Watson *et al.* 1990)
England	Suckler	7 flocks, 358 ewes	Subclinical	11.7%		(Watkins *et al.* 1991)
USA	Suckler	2 groups of 49 and 79 ewes	Subclinical		17-50% depending on methods	(Keisler *et al.* 1992)
Spain	Dairy	433 ewes	Subclinical		36.7%	(de la Cruz *et al.* 1994)
Greece	Dairy	760 ewes	Subclinical	10.9 %		(Fthenakis 1994)
Greece	Suckler	5 flocks	Subclinical	29-43%		(Stefanakis *et al.* 1995)
USA	Dairy	62 ewes	Clinical	29%		(Kirk *et al.* 1996)
Jordan	Suckler	12 flocks, 318 ewes	Subclinical	18.3%		(Al-Majali & Jawabreh 2003)
Greece	Experimental	2 flocks, 98 ewes	Subclinical	8.3%		(Fthenakis *et al.* 1998)

Location	Flock	Size of study	Mastitis classification	Prevalence	Incidence	Reference
Jordan	Mixed	46 flocks, 1736 ewes	NA	29.8% (subclinical)	1.7 cases per 100 ewe-months (clinical)	(Lafi *et al.* 1998)
Spain	Dairy	22 flocks, 564 ewes	Subclinical	34%		(Las Heras *et al.* 2002)
Iran	Dairy	178 ewes	Subclinical	39.0%		(Batavani *et al.* 2003)
Greece	Dairy	130 ewes	Clinical and subclinical	94.0%		(Fthenakis *et al.* 2004)
Greece	Dairy	140 ewes	Subclinical	5.7-15.6% (control group)		(Kiossis *et al.* 2007)
Norway	Mixed	353 flocks, 509 ewes	Clinical			(Mørk *et al.* 2007)
Canada	Suckler	30 flocks, 2792 ewes	Clinical		1.2%	(Arsenault *et al.* 2008)
Azerbaijan	Suckler	12 flocks, 260 ewes	Subclinical	9.2%		(Beheshti *et al.* 2010)
Italy	Dairy	2198 ewes	Clinical	75.0% (clinical)		(Marogna *et al.* 2010)

1.3. Risk factors associated with mastitis

Time in lactation is associated with mastitis; Fthenakis (1994) found that the prevalence of subclinical mastitis increased as the lactation period proceeded. However, the prevalence of subclinical mastitis is particularly difficult to measure as it depends on the degree of inspection given to an animal.

In suckler ewes, clinical cases often peak during the first week after lambing (Lafi 2006). In a study of 509 ewes in Norway, one third developed clinical mastitis in the first week. A second peak was also observed at three weeks (Mørk *et al.* 2007).

Reviewers have suggested that the rationale behind these peaks in cases of mastitis include;

1. Milk accumulation in the cistern at peak lactation may act as a reservoir for pathogens (Winter 2001).

2. Rapid lamb growth may cause lambs to nurse aggressively, butting the udder and damaging teats, leaving them susceptible to bacterial infection (Winter 2001).

Alternatively, the onset of lactation triggers mastitis because sheep may already have bacteria colonised in the udder, and when there is a change, either in the ewe or the environment, such as the onset of lactation, the infection becomes a detectable disease. It is also possible that functions of pregnancy and lactation are prioritised over the expression of immunity and hence reduced energy availability during these periods would compromise the immunity of the ewe (Coop & Kyriazakis 1999).

Larsgard and Vaabenoe (1993) following 920 ewes over 6 years, identified several factors associated with mastitis in Norwegian sheep including the number of lambs born, breed, udder conformation, type of pasture and milk yield (shown through growth of lambs). Whilst age did not have an effect on the risk of mastitis, affected ewes were culled before the next season, which may have biased the results (Larsgard & Vaabenoe 1993).

The two most recent articles to be published on risk factors for mastitis are also the most relevant, both focusing on suckler ewes. Despite the differences in country,

both produced similar results. Waage and Vatn (2008) identified individual animal risk factors for ovine clinical mastitis in suckler ewes in Norway. In 1056 flocks, the total number of lambs born per ewe, ewe age, breed, whether assistance at lambing was required, mastitis in a previous lactation and the presence and degree of teat lesions were identified as risk factors for mastitis (Waage & Vatn 2008). In a study of 2792 ewes from 30 commercial suckler flocks in Canada, the risk factors for clinical mastitis were region, ewe age, body condition score, whether ewes had had mastitis previously and the number of lambs born per ewe (Arsenault *et al.* 2008).

In agreement with these studies, it was suggested that ewes rearing more than one lamb are more likely to suffer from mastitis (Gross *et al.* 1978, Watkins *et al.* 1991). This may be a result of ewes rearing more than one lamb being more susceptible to lesions caused by lambs due to the cumulative stress on the mammary tissue (Lafi *et al.* 1998).

Several previous studies have identified a link between age and mastitis, with older ewes being more susceptible (Arsenault *et al.* 2008, Gross *et al.* 1978, Watkins *et al.* 1991). Damage to the udder from previous lambs might also make older ewes more susceptible to mastitis (Lafi *et al.* 1998).

It appears that there is a balance between ewe factors (predisposing conditions, existing trauma, lowered immunity and nutrition), the species of pathogen and the environment that affects the likelihood of the development of mastitis.

1.4. Aetiology of mastitis

Over 130 different organisms have been associated with infection of the bovine mammary gland (Watts 1988). It is probable that a similar number would be found in the ovine mammary gland. Many of the bacterial species that cause bovine mastitis can also be isolated from ovine mastitis cases. The complexity of mastitis is further highlighted when we consider not only species, but strain type. Strain types within a species can vary in pathogenicity and transmission pathway (Zadoks & Fitzpatrick 2009). The organisms are likely to come from the ewes' environment, such as other infected udders, replacement animals (contagious) and/or bedding, soil, water and manure (environmental).

In suckler ewes, bacteria commonly isolated from subclinical mastitis include coagulase-negative staphylococci (CNS), such as *Staphylococcus epidermidis, Staphylococcus simulans, Staphylococcus chromogenes,* and *Staphylococcus xylosus* (Fthenakis 1994). *Staphylococcus aureus* is also typically isolated from cases of subclinical mastitis in suckler ewes (Bergonier *et al.* 2003, Kiossis *et al.* 2007, Kirk *et al.* 1996, Mørk *et al.* 2007, Winter & Colditz 2002). Coagulase-negative staphylococci are also a common cause of subclinical mastitis in dairy ewes (Kirk *et al.* 1996, Mørk *et al.* 2007, Pengov 2001). Bacteria isolated from clinical mastitis cases include *Staphylococcus aureus, Mannheimia haemolytica, Escherichia coli, Mycoplasma* spp. (Bergonier & Berthelot 2003) and *Streptococcus* spp. (Fragkou *et al.* 2011, Fragkou *et al.* 2007c, Fragkou *et al.* 2007d, Las Heras *et al.* 2002) such as *Streptococcus uberis* (Marogna *et al.* 2010).

In addition, a variety of other bacterial species have been associated with mastitis including: *Actinomyces pyrogenes* (Saratsis *et al.* 1998), *Arcanobacterium pyogenes* (Fthenakis 1994, Fthenakis *et al.* 2004), *Bacillus* spp. (Batavani *et al.* 2003, Fthenakis 1994), *Burkholderia cepacia* (Berriatua *et al.* 2001), *Clostridium perfringens (Osman et al. 2009), Corynebacterium* spp. (Mavrogenis *et al.* 1995), in particular *Corynebacterium bovis* (Beheshti *et al.* 2010), *Klebsiella pneumoniae* (Bergonier & Berthelot 2003, Fthenakis & Jones 1990b), *Listeria monocytogenes* (Fthenakis *et al.* 1998), *Pseudomonas aeruginos* (Bergonier & Berthelot 2003) and *Serratia marcescens* (Tzora & Fthenakis 1998).

The differences in isolated mastitis causing pathogens could be due to differences in management practices between dairy and suckler ewes.

1.4.1. Environmental and contagious pathogens

Traditionally, mastitis pathogens have been classified as either environmental or contagious. However, the availability and reduced cost of molecular and DNA-based methods in mastitis research has resulted in an increased understanding of the transmission, reservoirs and persistence of bacterial strains that cause intramammary infections.

Much like the classifications of clinical and subclinical mastitis, these classifications are too rigid and not perfect. Some pathogens, traditionally thought of only as

contagious, can also be environmental in origin. These infections would not be affected by the implementation of a control and prevention program aimed at contagious pathogens, indicating the importance of strain typing studies (Zadoks & Fitzpatrick 2009). Different strains may differ in their ability to infect an udder, spread from ewe to ewe, alter somatic cell count, impact milk yield and respond to antimicrobial treatments.

1.5. The defensive role of the mammary gland

The mammary gland has anatomical features have could reduce the likelihood of pathogenic bacteria entering the teat duct. Sphincter muscles keep the teat canal tightly closed in order to prevent the entry of pathogens. The teat canal is lined with keratin, considered to be the first line of physical defence for the udder (Forbes 1970). It hinders the movement of bacteria up the teat canal and also contains antimicrobial agents (Sordillo & Streicher 2002). In an experimental study of dairy cows, where the keratin was partially removal from the teat canal, the ability of the teat canal to act as a protective anatomical feature against bacterial pathogens from the external environment was compromised (Capuco *et al.* 1992). In addition, the presence of induced subepithelial lymphoid tissue between the teat duct and teat cistern appeared to have a role in the protection of the mammary gland against the early stages of bacterial infection (Fragkou *et al.* 2010, Fragkou *et al.* 2007b).

Although not playing a defensive role, udder conformation can increase or decrease a ewes susceptibility to mastitis based on reducing damage to the udder. In a study of 2251 ewes, udder conformation was associated with the risk of mastitis and increased somatic cell count. As the cistern height increased and the degree of udder suspension (the weakness of the suspensory ligament and how loose and pendulous the udder was) and udder depth (the depth of the udder from the abdominal wall to the udder cleft when viewed from behind) decreased, the risk of mastitis increased (Casu *et al.* 2010).

Non-diseased mammary glands have bacteria colonising the udder and teat skin (Fragkou *et al.* 2007a), which may be a source of bacteria that could be transferred into the teat duct (Fragkou *et al.* 2011, Scott & Jones 1998). Interactions between the host's mammary defence system and the virulence of the invading pathogenic

bacteria determine the severity and extent of infection and disease. In order to understand the pathogenesis of a multifactorial microbial disease such as mastitis, it is important to consider the microbiota of the udder skin, as well as the bacterial content in the milk and in particular temporal interactions between them. It has been postulated that entrance of a pathogen into the teat would not necessarily result in infection (Mavrogianni *et al.* 2006a). For example, some bacteria may enter the teat canal but subsequently be withdrawn during suckling (Gougoulis *et al.* 2008b). In an experimental study, *Mannheimia haemolytica* inoculated into the teat duct did not always cause clinical infection suggesting the protective role of the teat (Mavrogianni *et al.* 2005). In the sequel to that work, using the same experimental design including ewes with natural or experimentally generated lesions had pathogenic bacteria deposited into the teat duct, which resulted in the development of mastitis, unlike those ewes in the previous study without lesions (Mavrogianni *et al.* 2006b). It was postulated that *Mannheimia haemolytica* may form part of the teat duct bacterial community without resulting in infection, suggesting that this bacteria does not necessarily always cause mastitis and for mastitis to occur, it requires either excessive accumulation of the bacteria (Fragkou *et al.* 2007a) or previous injury to the teat, lowering the teats bacterial defences in sheep (Mavrogianni *et al.* 2006b).

1.6. Sources & transmission pathways of pathogenic bacteria

There are three possible routes for bacteria to invade the mammary gland and cause infection: galactogenic route of entry (that is through the teat canal, with ascending infection), haemotogenous spread which would diffuse through mammary paranchyma, and percutaneous entry (wounds) (Meador 1988). There are also several reservoirs of bacteria that could cause mastitis, such as the lamb mouth, the ewe's udder skin or the environment, such as the bedding (Gougoulis *et al.* 2008b, Piccinini *et al.* 2009). Pathogenic bacteria are most likely to enter the udder half through the teat orifice, colonising the teat canal and cistern. Previous research on the transmission and persistence of mastitis causing bacteria from different environments of ewes is lacking.

1.6.1. Mouth & nasal cavities

Mannheimia haemolytica has been isolated from the mouths of ewes and lambs, and the teat skin of pregnant and lactating ewes, which may invade the mammary gland during suckling (Fragkou *et al.* 2011, Scott & Jones 1998) and has been indicated as being transmitted to the teat duct during suckling, along with staphylococci in a study on the microbiota of the teat before and after suckling in ewes (Gougoulis *et al.* 2008b). Although healthy teat ducts provide an effective defence for bacteria transmitted from the lamb's mouth to the teat duct of the ewe, suckling is likely to increase the risk of infection by increasing the teats exposure to bacteria (Gougoulis *et al.* 2008b). Despite this indication, strain typing methods were not used in these studies and therefore transmission could not be confirmed. In addition, providing evidence for the transmission of an isolate from one environment to another is difficult; there is the possibility for a strain to mutate as it moves which could only be detected by single nucleotide polymorphisms (SNPs) rather than methods typically used for strain typing. Therefore whilst you would expect isolates involved in transmission to be indistinguishable using strain typing methods, there is the possibility that they would not be, complicating the confirmation of a transmission pathway.

In sheep, identical *Staphylococcus aureus* pulsotypes (using pulsed-field gel electrophoresis as the strain typing method) were identified from body sites and in the milk. In particular the majority of pairs of isolates from the nasal cavity of ewes and their lambs, twins and repeated sampling provided identical pulsotypes indicating transmission of *Staphylococcus aureus* between the dam and her lambs (Mørk *et al.* 2012b). However, this could also be due to the predominance of a particular strain type within these flocks, and therefore does not necessarily indicate that a transmission event occurred. This is the only study indicating a transmission route of mastitis causing bacteria between the dam and her lambs using a strain typing method in suckler ewes.

1.6.2. The milking environment

Improper milking techniques can increase the likelihood of mastitis, due to the increased teat canal dilation time which allows bacteria to invade the teat (Gougoulis

et al. 2008b). Staphylococci have been thought to originate from milkers' hands or animal teat skin. Hand milking has been shown to increase the risk of bacterial colonisation of the teat, although only a small and non-significant increase in the risk of intramammary infection associated with hand milking has been shown (Mavrogianni *et al.* 2006c).

1.6.3. Bedding and dirt

Bedding type has an effect on the number of coliforms and *Klebsiella* spp. found on teat ends (Zdanowicz *et al.* 2004), which is to be expected as cows lie down on the bedding, allowing bacteria to be transferred from the bedding material to the teat skin. In addition, it has been suggested that pathogens could be transmitted to milk via dirty udders and teats in cattle (Vissers *et al.* 2007).

1.6.4. Udder skin

In dairy cows, potential reservoirs of *Staphylococcus aureus* have been identified; heifers with teat skin colonised by *Staphylococcus aureus* at parturition were more likely to have intramammary infections caused by *Staphylococcus aureus* at parturition (Roberson *et al.* 1994). In addition, using pulsed-field gel electrophoresis, the same pulsotypes of *Staphylococcus aureus* were identified from teat skin as in the milk in several studies (da Costa *et al.* 2014, Haveri *et al.* 2008, Zadoks *et al.* 2002). Pulsed-field gel electrophoresis has also been used to identify the same *Staphylococcus aureus* pulsotypes from body sites (particularly the hock skin), the immediate environment of lactating cows and milk samples previously (Capurro *et al.* 2010).

A study with extramammary sites including dairy cow teat skin, teat canals, skin lesions, milking liners and the hands and nostrils of milking personnel in two dairy herds found indistinguishable *Staphylococcus aureus* pulsotypes for isolates infecting the mammary gland and extramammary sites, again indicating potential reservoirs of intramammary infections (Haveri *et al.* 2008). However, the direction of transmission could not be ascertained.

1.6.5. Teat lesions

Teat bites could predispose intramammary infections due to removal of the acid mantle on the teat skin which could aid bacterial attachment (Sieber & Farnsworth 1984). In addition teat lesions could harbour bacteria, which could be transmitted up the teat canal during suckling. Authors have identified an association between *Arcanobacterium pyogenes, Streptococcus dysgalactiae* and teat lesions in cows (Ericsson Unnerstad *et al.* 2009).

1.6.6. Teat apices

Bacteria present on teat apices may have a different natural community to that of the rest of the udder due to suckling calves or lambs. The bacteria present on the teat duct may be advanced up the teat canal during suckling, unlike bacteria present on the udder. The teat duct could harbour bacteria, which could invade the mammary parenchyma, causing mastitis (Mavrogianni *et al.* 2006a).

Klebsiella, streptococci and staphylococci have been isolated from the teat ends of dairy cows (Rendos *et al.* 1975). *Lactococcus* spp., yeasts and *Pseudomonas* spp. have also been isolated from dairy cow teats and udders, although there was no correlation between the level of *Lactococcus* spp. and yeasts in milk and on the teats and udders (Desmasures *et al.* 1997). Supré *et al* identified the same *Staphylococcus devriesei* strain from teat apices and the milk of dairy cows (Supré *et al.* 2010).

In a study of 11 ewes, *Mannheimia haemolytica* was isolated from the teat duct only after suckling, indicating it had been transmitted to the teat duct during suckling by the lamb (Gougoulis *et al.* 2008b).

Bacteria such as *Staphylococcus chromogenes, Staphylococcus hyicus, and Staphylococcus aureus* have been isolated from teat canal keratin and milk secretions. In addition, quarters from cows with teat canal colonisations of bacteria without a clinical intramammary infection had a higher somatic cell count than quarters from cows without teat canal colonisation and intramammary infections, suggesting the importance of teat canal colonisation on the health of the mammary gland (Trinidad *et al.* 1990).

1.6.7. Other potential reservoirs of mastitis-causing pathogens

Strains of *Staphylococcus aureus* predominant on some farms were isolated from flies collected in the barns of lactating cows and milking equipment (Capurro *et al.* 2010).

The infected udder gland is also a reservoir for transmission between individual udder halves (Mørk *et al.* 2007, Vautor *et al.* 2003).

Although these studies provide accumulating evidence for reservoirs and transmission routes of pathogens associated with intramammary infections, molecular methods at the subspecies level over time are required in order to ascertain whether the isolates are the same strain type, therefore proving a transmission event has occurred.

1.7. The protective role of bacterial species

In recent years, the potential protective role of coagulase-negative staphyloccci in mastitis in dairy cows has been a subject of debate, with contradictory results having been published. Some authors suggest that quarters infected with minor pathogens, such as coagulase-negative staphylococci are preventive for natural infections by major pathogens. (Rainard & Poutrel 1988, White *et al.* 2001). For example, in a challenge study, protective effects against *Staphylococcus aureus* infection were found using *Staphylococcus chromogenes* (Matthews *et al.* 1990). *In vitro* studies have supported this, showing the inhibition of *Staphylococcus aureus* and streptococci growth by *Staphylococcus chromogenes* (De Vliegher *et al.* 2004). Furthermore, some strains of coagulase-negative staphylococci were shown to inhibit the growth of *Streptococcus agalactiae*, possibly due to increased somatic cell count in the milk or antibacterial peptides produced by the bacteria (dos Santos Nascimento *et al.* 2005, Pyörälä & Taponen 2009).

Conversely, studies have found that heifers infected with coagulase-negative staphylococci pre-calving increased the risk for post calving infection with coagulase-negative staphylococci, *Staphylococcus aureus* or *Streptococcus uberis* (Compton *et al.* 2007, Parker *et al.* 2007). Alternatively, one study has shown that quarters infected with *Staphylococcus* spp. were less susceptible to experimental

challenge with certain species, such as *Staphylococcus aureus*, but more susceptible to *Streptococcus agalactiae (Nickerson & Boddie 1994)*.

Finally, some studies have shown no protective or predisposing role of coagulase-negative staphylococci in mastitis caused by *Staphyloccocus aureus*, or *Streptococcus uberis* (Hogan *et al.* 1988, Zadoks *et al.* 2001).

1.8. Persistence of infections in the mammary gland

Whilst a great deal of research has identified sources of infection, little research has been done on the persistence of particular strains in the milk throughout lactation in cows once bacteria have penetrated the mammary gland. In addition, much of the research on ovine mastitis still leverages on bovine mastitis research. There is no research on the persistence of infections in sheep during the lactating or dry period. Different strains within a bacterial species can differ in pathogenicity and transmission routes, and therefore classification of isolates at the species level can incorrectly oversimplify control measures recommended, hence the need for longitudinal strain typing methods.

1.8.1. Infection persistence during lactation

Recent studies have used strain typing techniques such as pulsed-field gel electrophoresis to isolate the same strain of bacteria from the same mammary quarter on more than one occasion indicating the persistence of the same infection over time with coagulase-negative staphylococci based on a study of 12412 milk samples from 3 dairy research herds (Gillespie *et al.* 2009). In fact, coagulase-negative staphylococci were shown to persist for up to 10 months (Gillespie *et al.* 2009). *Escherichia coli* has also been shown to persist in the bovine mammary gland in a study of 300 dairy cows, with an estimated occurrence of between 4.8% and 9.1% (Döpfer *et al.* 1999, Lam *et al.* 1996). In addition the occurrence of recurrent episodes of the same strain in any quarter in a cow is high (Döpfer *et al.* 1999), suggesting transmission between quarters, which could increase persistence of the strain. Lipman *et al.,* (1995) also found persistence of the same *Escherichia coli* serotypes in the bovine mammary gland, although different methods (serotyping and DNA polymorphism patterns) were used. In addition, infection with *Escherichia coli* more than once in a lactation was infrequent (Lipman *et al.* 1995). Conversely, in a

study of 503 cows from 5 herds, quarters were often infected with multiple *Streptococcus uberis* strain types, despite the ability of *Streptococcus uberis* to persist in the udder (McDougall *et al.* 2004). This suggests that some pathogens have a superior mechanism of bacterial persistence than others. In fact, some pathogens such as *Listeria moncytogenes,* are able to persist in milking parlor environments which could be a source of reinfection (Ho *et al.* 2007).

The ability of *Staphylococcus aureus* to produce biofilm could be why mastitis associated with this species of bacteria so often becomes chronic and persists. The role of exopolysaccharride (slime) in the colonization and virulence in bovine mastitis has been shown in cows (Cucarella *et al.* 2004). It is thought to be involved in the enhancement of initial attachment to epithelial cells and in the subsequent formation of micro-colonies, as has been shown for *Escherichia coli* strains (Chan *et al.* 1982).

1.8.2. The role of the dry period

The bovine mammary gland is thought to be particularly susceptible to environmental coliform and streptococcal infections during the dry period (Larry Smith *et al.* 1985, Oliver & Mitchell 1983, Todhunter *et al.* 1995), including *Streptococcus uberis* (Todhunter *et al.* 1995) and *Escherichia coli* (Bradley & Green 2001, Döpfer *et al.* 1999, Lipman *et al.* 1995). Experimental studies have shown the ability of pathogens to remain within the udder, causing clinical disease after the onset of lactation (Bradley 2002, McDonald & Anderson 1981).

Two studies in particular highlight the significance of the dry period in the persistence of mastitis causing pathogens.

In a study of 629 cows from 6 commercial herds, samples were collected during the dry period and from clinical quarters of these cows during the subsequent lactation allowing comparisons to be made between these time periods. DNA fingerprinting showed the persistence of Enterobacteriaceae acquired during the dry period, causing disease after the onset of lactation (Bradley & Green 2000). One quarter remained persistently infected for >200 days before resulting in severe clinical mastitis (Bradley 2002). Of all the enterobacterial mastitis occurring in the first 100 days of lactation, 52.6% arose in quarters previously colonised with the same strain of

bacteria during the dry period (Bradley & Green 2000). This suggests that chronic infections should be considered and environmental management during the dry period is imperative in the control of environmental mastitis.

Bradley and Green (2001) observed 6 commercial herds over a period of 12 months in order to identify changes in the behaviour of *Escherichia coli*. DNA fingerprinting allowed the identification of the genotypes of strains involved in recurrent cases of clinical *Escherichia coli* mastitis. In the majority of cases, the same genotype was implicated as the cause of disease in recurrent cases and often the same genotype was identified in different quarters of the same cow suggesting that the same genotype may persist in the mammary environment (for in excess of 100 days), causing recurring infections and that bacteria may be spread between quarters (Bradley & Green 2001).

PCR based DNA fingerprinting identified the same *Streptococcus uberis* and *Streptococcus dysgalactiae* strain types from the same infected mammary glands from one lactation to the next, highlighting the persistence of these organisms through the dry period and during lactation (Oliver *et al.* 1998).

Statistical modelling has been used to identify the relationship between intramammary infections during the dry period, and clinical mastitis in the next lactation. The probability of an udder quarter developing clinical mastitis increased when *Streptococcus dysgalactiae, Enterococcus faecalis, Escherichia coli,* or *Enterobacter* spp. were cultured at drying off. In addition, the risk of clinical mastitis caused by specific pathogens increased if they were cultured in 2 or more late dry and post calving samples. Interestingly, the time that an isolate was identified was important in whether it increased or decreased the risk of clinical mastitis development; *Corynebacterium* spp., when isolated at drying off were associated with an increase, but when isolated in the late dry or post calving samples was associated with a reduction in the risk of clinical mastitis (Green *et al.* 2002). Although this study did not use strain typing to confirm the persistence of certain strains in the mammary gland, it provides evidence for the significance of the dry period on clinical mastitis development.

1.9. The importance of strain typing

Throughout this review, strain typing has been shown to be important in studies of reservoirs, transmission and persistence. However the importance of strain typing is also highlighted when host responses are considered. Host responses to intramammary infections differ between pathogen species and strains (Zadoks & Fitzpatrick 2009). In particular, *Staphylococcus aureus* is particularly difficult to control due to its wide pattern of virulence factors such as leukotoxins, which can selectively destroy host polymorphonuclear leukocytes (PMN) and monocytes and secretion of exopolysaccharides and thermostable toxins (Craven & Williams 1985). As many as 30 extracellular products are produced by *Staphylococcus aureus* (Rogolsky 1979). This includes several toxins that contribute to the pathogenesis of mastitis (Rogolsky 1979). There also appears to be differences between the toxins produced by strains of *Staphylococcus aureus* that are isolated from ewes with subclinical mastitis and clinical mastitis. For example, toxins produced from *Staphylococcus aureus* isolates from sheep with subclinical mastitis were less enterotoxigenic than those from acute clinical cases (de Santis *et al.* 2005, Hamann 2005).

1.10. Methods used to understand mastitis

1.10.1. Molecular epidemiology

In order to study disease in populations and identify factors that determine its occurrence, the integration of molecular biological techniques and epidemiologic research is required.

Epidemiological research identifies incidence, prevalence and patterns of disease at the population level. A longitudinal study can be used to investigate an outcome and exposure variables over a period of time. Such studies are particularly useful to study the progression to an outcome of interest over time, providing temporal associations between the outcome and exposure variables. Causal associations can be deduced more robustly in a longitudinal study, with statistical evidence for these associations (Hill 1965). Such studies are also flexible and can test a variety of hypotheses simultaneously. However, longitudinal studies are expensive and time-consuming. In

addition, data that are collected repeatedly over time from the same individual result in clustered data that need to be managed during analysis.

Different strains within a bacterial species can vary in terms of reservoir, transmission pathway, persistence and pathogenicity. Strain typing is therefore necessary to identify sources and transmission routes of mastitis-causing bacteria and to choose appropriate prevention and treatment measures. Although contributing to our understanding of mastitis, molecular epidemiological studies have not yet reached a stage where strain typing is time and cost effective enough to be used in real time to contribute to outbreak analysis and prevention and/or treatment options. Ideally, a method of strain typing that is rapid and cost effective is required in order for large numbers of samples from numerous animals to be analysed.

1.10.2. Laboratory based techniques

Culturing of bacteria

There are a variety of methods used to identify mastitis causing pathogens. Traditional culture techniques are often the first step in identification of an isolate, in order to culture pure growth. Biochemical tests and Gram stains are often used to identify bacteria to the genus or species level.

Polymerase Chain Reaction (PCR) amplification

In the past, commercial kits differentiated species of bacteria based on specific biochemical reaction characteristics of each. These methods, however, were based on phenotypic reactions and often failed in the identification of different species of coagulase-negative staphylococci (Heikens *et al.* 2005). In order to overcome these issues, alternative identification methods based on molecular rather than phenotypic characteristics were explored. Several genomic targets have been used for the molecular identification of different bacterial species, most commonly the genes for 16s rRNA (Onni *et al.* 2010).

The majority of mastitis causing pathogens in cows can now be detected based on PCR which detect the main species-specific DNA sequences. Techniques have been developed to identity a single species (uniplex) or multiple species simultaneously

(multiplex) (Gillespie & Oliver 2005, Phuektes *et al.* 2001, Riffon *et al.* 2001). Recent advances have resulted in a multiplex PCR to be developed for the simultaneous detection of up to 10 bovine mastitis pathogens (Shome *et al.* 2011) and is now being used to detect pathogens found in bulk tank milk samples (Boss *et al.* 2011).

In sheep, coagulase-negative staphylococci have been identified from ovine milk samples via PCR amplification of the staphylococcal 16S rRNA and *gap* genes (Onni *et al.* 2010) whilst *Staphylococcus aureus* has been identified via PCR-Restriction Fragment Length Polymorphism Analysis of the *aroA* Gene (Marcos *et al.* 1999).

Both the biochemical and PCR methods described above can identify isolates to species level in some cases. However in order for multiple isolates to be identified to strain level, the following methods are most often used.

Pulsed-field gel electrophoresis (PFGE)

Pulsed-field gel electrophoresis is a method of typing an organisms based on fragment patterns produced by cleaving chromosomal DNA with restriction endonucleases. The bacterial genome is digested with a restriction enzyme that has relatively few recognition sites; generating fragments from 10-2000kb on the agarose gel unlike traditional gel electrophoresis whereby fragments of 0.5-25kb are resolved. Pulsed-field gel electrophoresis makes use of a current being applied in three directions to three electrodes, resulting in a higher level of fragment resolution (Vautor *et al.* 2005b).

Multilocus sequence typing (MLST)

Multilocus sequence typing (MLST) involves sequencing fragments of housekeeping genes (most commonly 7 housekeeping genes) from different strains of the same organism and comparing them. For each gene, a sequence of approximately 450bp is amplified (PCR) and then sequenced, which is presented in a dendrogram. Alleles at each locus based on nucleotide sequence are assigned. Multilocus sequence typing could be used to track a virulent strain of a bacterial pathogen as it moves through a population (Vautor *et al.* 2005b).

Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS)

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) is a relatively novel ionization technique which allows the analysis of biomolecules. Co-precipitation of a UV-light absorbing matrix and a biomolecule is vaporized by a laser pulse. The matrix becomes ionized when it absorbs most of the laser energy (preventing unwanted fragmentation of the biomolecule). The energy is 'passed from the matrix' to the vaporized protein molecules which are accelerated in an electric field, drawn into the evaporation chamber into the mass spectrometer and are separated according to their mass to charge ratio by reaching the detector at different times. Each molecule yields a distinct signal (De Bruyne *et al.* 2011).

MALDI-ToF-MS, due to its speed and low running costs, has the potential to replace conventional phenotypic identification. In addition, several recent studies have indicated MALDI-ToF-MS could be used for rapid strain differentiation and identification for *Escherichia coli* (Christner *et al.* 2014, Karger *et al.* 2011), *Yersinia enterocolitica* (Stephan *et al.* 2011), *Salmonella enterica* (Dieckmann & Malorny 2011), *Clostridium difficile* (Reil *et al.* 2011), *Staphylococcus aureus* (Boggs *et al.* 2012, Wolters *et al.* 2011), *Propionibacterium acnes* (Nagy *et al.* 2013), *Listeria* spp. (Barbuddhe *et al.* 2008), *Leptospira* spp. (Rettinger *et al.* 2012), *Saccharomyces cerevisiae* (Moothoo-Padayachie *et al.* 2013), Mycobacteria spp. (Hettick *et al.* 2006), and *Arthrobacter* spp. (Vargha *et al.* 2006).

Due to the application of this method to strain typing being very recent, it is not typically used for strain typing studies and has not been used to support epidemiological studies.

1.10.3. Epidemiological techniques

'Epidemiology is the study of disease in populations and of factors that determine its occurrence' (Thrusfield 2013).

Cross-sectional studies

Cross-sectional studies allow the examination of the relationship between disease prevalence and exposure at a single point in time in a given population. The cross-sectional study in this thesis utilises a questionnaire. Questionnaires are particularly useful when there is no previous information readily available on a subject. They are designed to record information in a standardised format and the aim is to achieve a high response rate. The advantages of using a postal questionnaire include a wide coverage of a population and the fact that they are quick to administer and avoid interview bias. However, there is no way to check if a question is misunderstood. A pilot study can ensure that questions are understood, and if not feedback can expose defects in the questionnaire that can be amended (Thrusfield 2013).

The advantages of using a cross-sectional study to examine the risk factors of mastitis include the large population size. As the questionnaire is sent to a large number of farms with different management practices, a whole array of data can be obtained. The large sample, and the large amount of data obtained from this population can produce a wealth of information on the study of risk factors. However, often questionnaires, as in the current study, collect information retrospectively. This can result in less accurate information as respondents will not always remember management practices/detailed information about their farm and flocks over a year ago. However, these studies are hypothesis generating rather than testing cause and effect associations.

Longitudinal studies

A longitudinal study can utilise a group of individuals exposed to a particular disease compared with an unexposed group by observing each for a new disease, in this case an episode of mastitis. In this thesis, a cohort of ewes is followed and samples taken over time. Despite the potentially long duration for follow-ups and the expense,

cohort studies are flexible and can be used to test a variety of hypotheses simultaneously.

1.11. Thesis aims

Based on the findings of the literature, the overall aim of this thesis was to improve our understanding of the development and persistence of intramammary infections.

In order to address this aim, the following objectives were implemented:

1. To identify management practices that might act as risk factors for clinical mastitis (Chapter 2)
2. To investigate the use of MALDI-ToF-MS as a diagnostic tool for species identification and strain differentiation in order to:
    a. Identify reservoirs of mastitis causing pathogens on the skin of ewes (Chapter 3)
    b. Observe the carriage of these bacteria into the udder, which might lead to mastitis and identify whether bacteria persist over time in the mammary gland (Chapter 4)
3. To investigate whether udder and teat conformation are heritable traits that affect mastitis (Chapter 5)

Chapter 2. Risk factors for ovine clinical mastitis: a cross-sectional study of 329 farms in England

2.1. Introduction

Mastitis is an inflammation of the mammary gland usually caused by bacterial infection (Khan & Khan 2006). In suckler ewes (ewes rearing lambs for meat), mastitis may be acute, when the ewe shows signs of local or systemic disease such as a hot or cold mammary gland, lameness, or not eating supplementary food, or chronic, when masses in the mammary gland are detected by palpation during routine checks e.g. at weaning or before mating.

Mastitis results in direct and indirect economic losses for the suckler sheep industry. Costs arise from ewe and lamb deaths, culling chronically diseased ewes (Conington *et al.* 2008), ewe replacements and decreased live-weight gain in lambs reared by affected ewes (Fthenakis & Jones 1990a, Huntley *et al.* 2012, Keisler *et al.* 1992, Saratsis *et al.* 1998). An accurate estimate for the cost of mastitis to the UK sheep industry across all breeds is not available, however, a model indicated reducing the risk of mastitis by 10 per cent in Texel flocks would save £8.40 per ewe (Conington *et al.* 2008).

An estimate of the incidence rate of clinical mastitis (IRCM) depends on a farmer's ability to detect (frequency and attentiveness of observations) and record clinical cases of mastitis. There are no estimates of the IRCM of suckler ewes in the UK. The only available estimate outside the UK is from Canada, where it was estimated to be 1.2% p.a. (0 to 6.6%) (Arsenault *et al.* 2008).

In suckler sheep, clinical cases of mastitis have been reported to peak in the first week post-partum, with a second peak being reported at 3-4 weeks of lactation in Norway (Mørk *et al.* 2007) and at 4-7 weeks of lactation in Ireland (Onnasch 2000).

In dairy cows, the peak IRCM is in the first week of lactation (Olde Riekerink *et al.* 2008, Waller *et al.* 2009). One explanation for this is that there is a pre-existing bacterial infection in the mammary gland that develops into clinical disease after the onset of lactation (Bradley & Green 2000). Sheep also have bacteria present in the

mammary gland without signs of disease (Huntley *et al.* 2012). As a consequence, risks for infection might not be closely related temporally to disease events, however, risks that trigger disease might be temporally close to the disease event, for example a change in the ewe (such as the onset of lactation) (Kehrli *et al.* 1989, Oliver & M. 1988) or the environment (such as housing or lambs sucking). Alternatively new bacterial infections might occur in the first week of lactation due to the opening of the teat orifice combined with contaminated bedding and lambs sucking and cross-sucking, possibly transmitting bacteria from udder skin or the environment to the teat canal and between ewes.

Several studies outside the UK have identified risk factors associated with mastitis in suckler ewes. Risks included litter size, breed, udder conformation, pasture type, lamb growth rate, assistance at lambing, whether the ewe had mastitis in a previous lactation, ewe age, geographical region, ewe body condition (Arsenault *et al.* 2008, Gross *et al.* 1978, Lafi *et al.* 1998, Larsgard & Vaabenoe 1993, Waage & Vatn 2008, Watkins *et al.* 1991). In the UK, poor udder conformation and age were associated with high somatic cell count (Huntley *et al.* 2012).

Differences in management practices (such as indoor or outdoor lambing) are likely to be associated with variations in IRCM and exposures.

2.2. Aims

The aims of the current study were to estimate the incidence rate of clinical mastitis and identify potential management risk factors for clinical mastitis using a retrospective cross sectional postal study of a random sample of English sheep farmers.

2.3. Materials and methods

2.3.1. Study population

The number of sheep holdings in England in the 2003 census was 45,801 (DEFRA 2003). Based on this, the sample size required was 385, assuming at least 50% of flocks had at least one case of clinical mastitis, with 95% confidence and 90% power (Win-Episcope-2 2010). Assuming a response rate of 30% (Kaler & Green 2008),

999 farmers whose details were provided by EBLEX, the levy body for English sheep and beef farmers, were contacted in January 2010.

2.3.2. Design of the questionnaire

Published literature and veterinary expertise on risk factors for mastitis in sheep and cattle were used to design the postal questionnaire (Cooper 2011). Questions were based on the farm, flock, ewes, management regimes, mammary gland health, nutrition and housing. There were a total of 114 questions. The majority of questions were closed or semi-closed, however, there were some open questions, these included whether farmers thought that their ewes got clinical mastitis in certain fields, whether the farmer had changed farm management between 2008 and 2009 and opinions on the causes of mastitis and preventive actions.

2.3.3. Pilot study

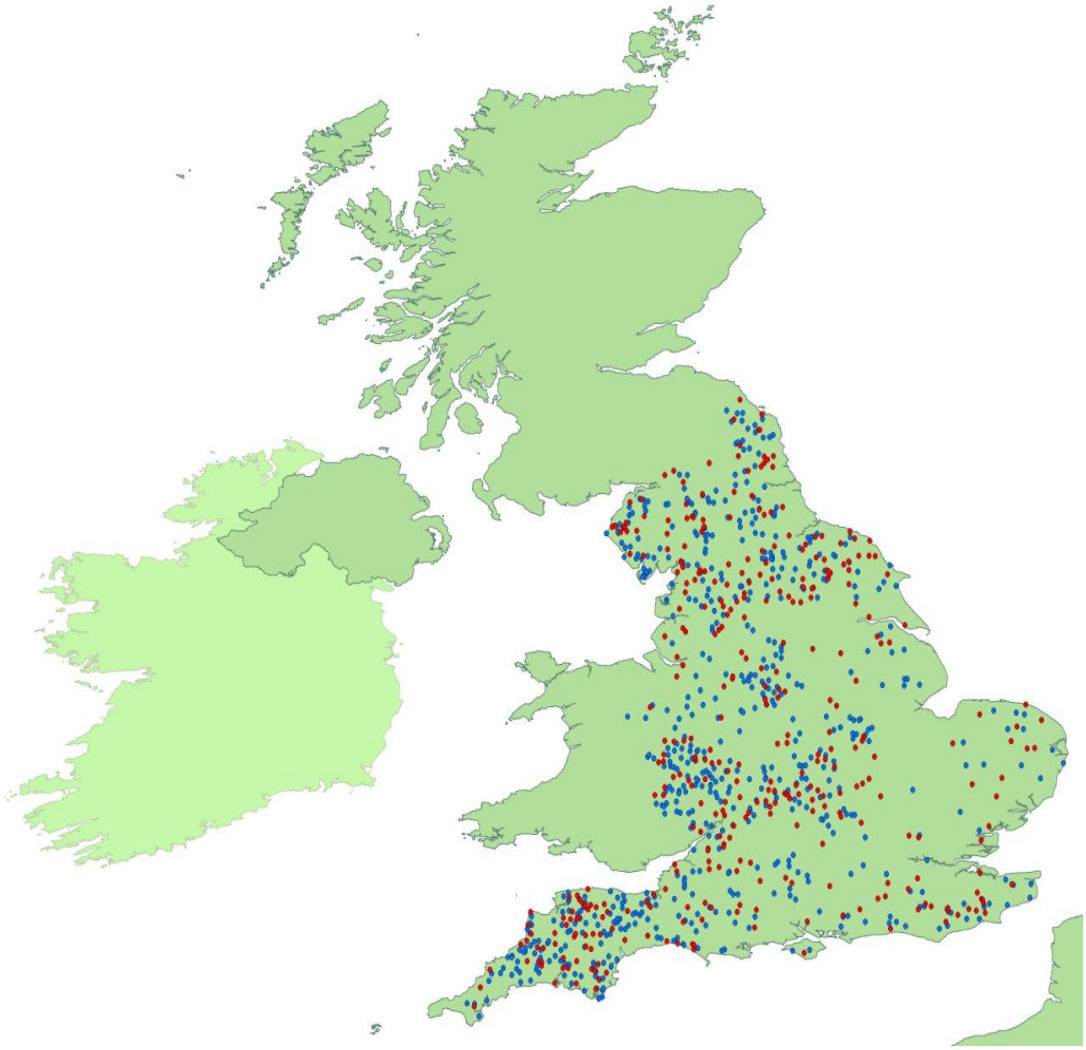
The pilot questionnaire was sent to 12 convenience selected farmers in the north of England that included commercial and pedigree flocks situated in lowland, hill and upland areas with between 50 and 1000 sheep. As a result of the feedback from the pilot study several additional questions were added to the questionnaire, and questions that had poor response rates or were answered incorrectly were re-designed.

2.3.4. Data collection & storage

The final questionnaire was sent out on 8th January 2010, a reminder was sent to non-respondents on 10th February 2010 and a second reminder and a second copy of the questionnaire were sent to non-respondents on 21st April 2010.

A database was designed in Microsoft Access 2007. Data were entered using multiple choice drop down boxes and checked. The postcodes from the 999 farmers were transformed into X and Y co-ordinates and inputted into ArcView with the worldwide shapefile from the Economic and Social Research Institute (ESRI) to create a map of respondents and non-respondents (Figure 2.1).

Figure 2.1: The location of respondents (red) and non-respondents (blue) in England.



2.3.5. Data analysis

Measures of dispersion and central tendency were used to investigate the data (R Core Team 2013). Normality was tested using Shapiro-wilks test and the arithmetic or geometric mean was calculated for variables in R. Obvious errors were corrected, and categories within variables with <5 responses were merged where logical. Queries were used to select and link data from related databases in Microsoft Access to select data for statistical analysis. Respondents with ≤20 ewes in their flock were removed from the analysis (n=4). Analysis of variance (ANOVA) was used to test the differences between group means in R.

The incidence rate of clinical mastitis (IRCM) per flock was calculated. The variance was greater than the mean and so over-dispersed Poisson regression models, offset

by flock size, were used to investigate factors associated with IRCM. A total of 144 variables were used to investigate management from 8 weeks before lambing, during lambing and during lactation. Farmers managed sheep either wholly indoors or outdoors or a combination of both, as a consequence 6 separate models were necessary. Model 1 included all respondents and covered general information about the farm, flock, lambing, mastitis, health management and nutrition. Model 2 included flocks housed in barns from up to 8 weeks before lambing to lambing. Model 3 included flocks housed during lambing, and Model 4 included flocks housed after lambing. Model 5 included flocks outdoors during lambing, and Model 6 included flocks outdoors after lambing. Outliers were assessed to determine their impact on the coefficients.

The models took the following general form:

$$g(E(Y) = \beta_0 + \sum \beta_m x_m - \log(O_i)$$

...where g is the log-link function, E(Y) the expected values of the outcome variable Y (the number of ewes with clinical mastitis in 2009), β_0 the intercept and βm the regression coefficients (expressing effects of the included predictor variables x_m), O_i as the offset (the number of ewes in the breeding flock in 2009).

Risk ratios were exp(β). To estimate the 95% confidence interval for exp(β), the standard error (S.E.) was calculated as the two-sided confidence coefficient assuming a normal distribution and multiplied by the standard error from the model: exp(β) ± 1.96 × S.E.

2.4. Results

2.4.1. Response rate

Of the 999 questionnaires sent out, 372 were returned (37.2% response rate), 329 of which were usable. The remaining analysis only included the 329 respondents who gave a response to both the number of ewes with clinical mastitis in 2009 and the number of breeding ewes in the flock in 2009.

2.4.2. Descriptive analysis

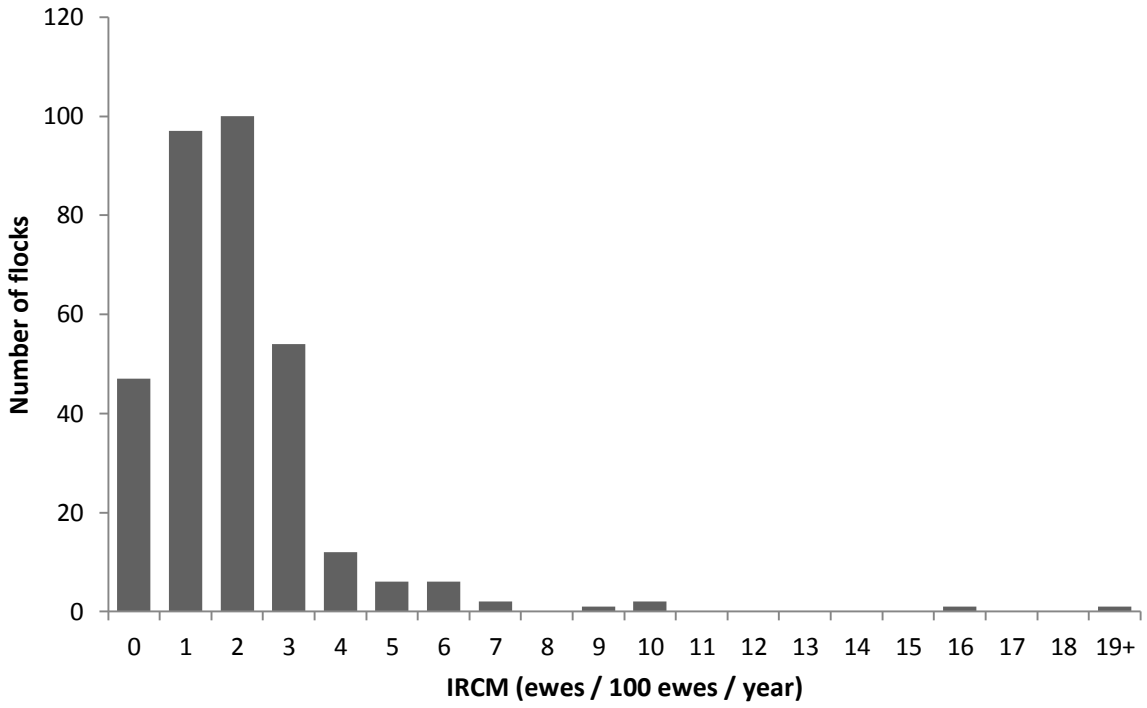
Farm characteristics

The majority of flocks were lowland (90.3%), and commercial (66.6%). The geometric mean number of breeding ewes / flock was 248.7 (range=21-4252, S.E= 24.8). Flocks were mostly comprised of ewes that were between 2 and 5 years of age. The geometric mean number of lambs reared per ewe across all flocks was 1.2 (S.E=0.3), which did not significantly vary by management practice.

Incidence rate of clinical mastitis

The geometric mean IRCM across all flocks was 1.2 / 100 ewes / year (range=0.0-19.0, var=3.6, S.E=0.10). Only 14.3% of respondents had no ewes with clinical mastitis in their flock (Figure 2.2).

Figure 2.2: The number of flocks by incidence rate of clinical mastitis in England.



The mean IRCM by flock type ranged from 0.82-1.98 depending on management practices; flocks that were always housed had significantly higher (p<0.01) IRCM than flocks that were never housed using ANOVA tests (Table 2.1). The mean

IRCM was 1.98 for ewes always housed indoors, 0.87 for ewes outdoors and 1.32 for ewes that were both outdoors and indoors.

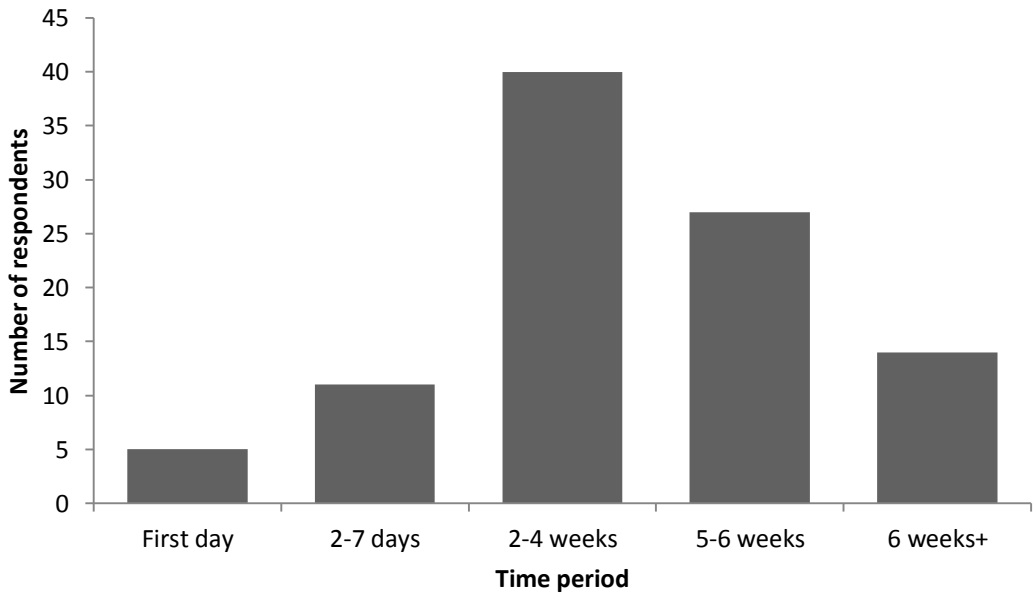
Table 2.1: The mean incidence rate of clinical mastitis by stage of production and ewe location.

		IRCM (/100 ewes/year)			
		Stage of production			
		Before lambing	At lambing	After lambing	Always
Ewe location	Outdoors	0.87	0.82	0.99	0.87
	Both	NA	1.25	1.41	1.32
	Indoors	1.39	1.41	1.82	1.98

Clinical mastitis

Ninety-one respondents (27.7%) reported a peak in mastitis cases. Some respondents reported > one peak (*n*=19). Respondents most frequently saw a peak 2-4 weeks after lambing (Figure 2.3).

Figure 2.3: The number of respondents that reported a peak in mastitis cases by time period.



The geometric mean percent of ewes with clinical mastitis that no longer had a functioning mammary gland half due to clinical mastitis was 34.4% (*n*=261,

S.E=2.5). The geometric mean percentage of the flock that died due to clinical mastitis in 2009 was 0.2% (*n*=268, S.E=0.04). On average, 3.1% of ewes that had mastitis died (*n*=261, S.E=1.4).

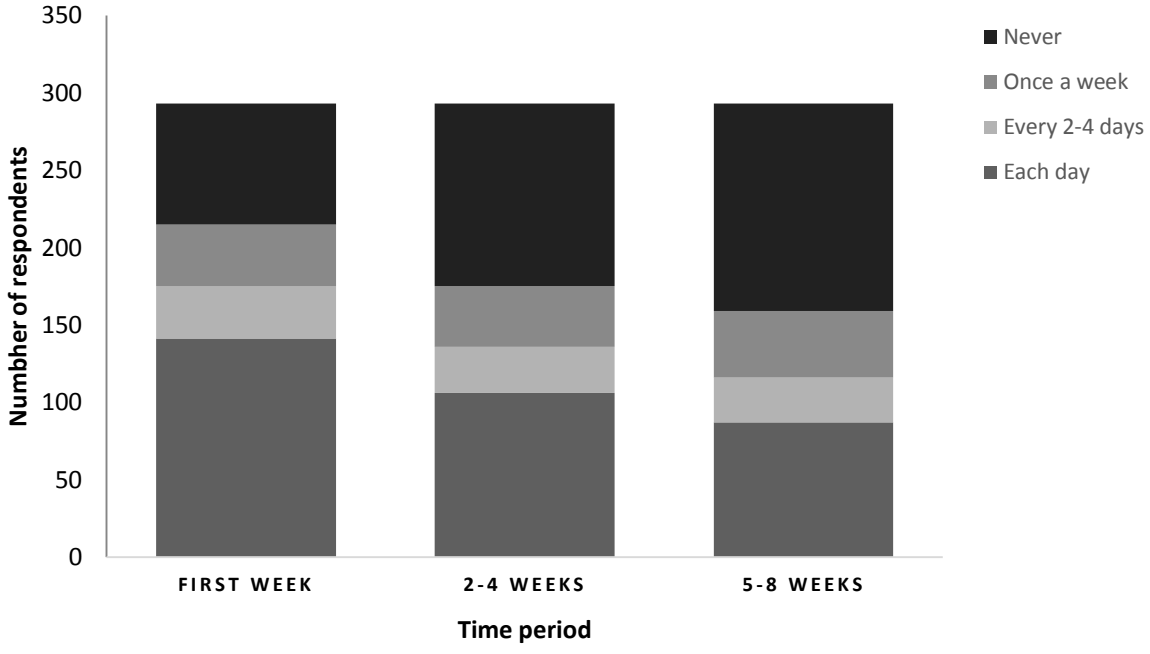
The majority of respondents did not keep ewes that had had mastitis previously (94.2%, *n*=180) and did not breed from ewes with clinical mastitis (92.4%, n=266).

The most popular method of treatment for clinical mastitis was antibiotic injection (92.1%, *n*=278), 33.1% (*n*=100) used intramammary antibiotics and the remaining 18.2% (*n*=55) used an anti-inflammatory.

Management practices

A total of 88.0% (287/326) of respondents checked ewes' mammary glands for function and disease at lambing. Less than 50.0% of respondents checked ewes each day in the first week of lactation and this percentage reduced further by 5-8 weeks into lactation. There were some respondents that never checked their ewes for mastitis: in the first week after lambing (26.6%), 2-4 weeks after lambing (40.3%), and 5-8 weeks after lambing (45.7%) (Figure 2.4).

Figure 2.4: The frequency at which respondents checked ewes' mammary glands in the first week after lambing, 2-4 weeks and 5-8 weeks after lambing.



Mammary gland abnormalities

Of those respondents that checked the udder at lambing, the geometric mean percent of flock with teat lesions was 0.9% and ewes with at least one blind ending teat was 0.8%. The geometric mean percent of ewes per flock with poor udder conformation was 1.3% (S.E=0.2) (*n*=173).

Within the mastitis section of the questionnaire, there were questions about the numbers of ewes with mammary gland abnormalities, and the proportion of these ewes that were culled before tupping and at weaning (Table 2.2). Of the 281 respondents that had at least one ewe with mastitis between weaning 2008 and tupping 2009, 127 completed this section. On average, there was a slightly higher percentage of the flock with mammary gland abnormalities at weaning compared to before tupping (Table 2.2), except for ewes with teat cords (hard fibrous structure identified by palpating the teat), where more of these mammary gland abnormalities were observed before tupping than at weaning (Table 2.2). Respondents managed different abnormalities differently; for example respondents culled on average between 85.6% and 93.1% of ewes with clinical mastitis at weaning and before tupping respectively whereas a smaller percentage of ewes affected with teat warts were culled at weaning and before tupping compared to other mammary gland abnormalities (31.79% and 29.94% respectively) (Table 2.2).

Table 2.2: The mean percentage of flock affected and culled with different mammary gland abnormalities.

	Mean percentage of flock affected with abnormality		Mean percentage of flock culled with abnormality		Mean percentage of ewes with abnormality culled	
	Before tupping	Weaning	Before tupping	Weaning	Before tupping	Weaning
Mass in mammary gland	0.55	0.68	0.50	0.60	90.87	87.58
Clinical mastitis	0.22	0.34	0.20	0.29	93.11	85.58
Teat cord	0.16	0.12	0.13	0.10	81.59	79.44
Teat skin damage	0.04	0.05	0.02	0.02	43.72	50.33
Teat warts	0.09	0.14	0.03	0.05	29.94	31.79

2.4.3. Risk factors for clinical mastitis

The significant univariate associations between exposures and IRCM are presented in Tables 2.3 and 2.4.

Model 1 – General flock management

As the percentage of the flock with poor udder conformation increased, the IRCM increased (*RR: 1.11; CI: 1.05:1.16*) (Table 2.5). Lambing some (*RR: 0.28; CI: 0.09:0.89*) or all ewes (*RR: 0.01; CI: 0.00:0.18*) outdoors was associated with a decreased IRCM compared with lambing ewes indoors (Tables 2.1, 2.5). As the mean number of lambs reared per ewe in the flock increased the IRCM increased. There was an interaction between the number of lambs reared per ewe and ewes lambed indoors; for flocks lambed indoors, as the number of lambs per ewe increased, the IRCM decreased. Conversely, for flocks that were lambed partly or wholly outdoors as the number of lambs per ewe increased, the IRCM also increased (*RR:2.5, CI: 1.12:5.44, RR: 13.89; CI: 2.14:90.03*) (Table 2.5). In the remaining models, the percentage of flock with poor udder conformation was forced into the model.

Model 2 – Ewes housed in barns before lambing

The base material of the floor in the barn before lambing was significantly associated with the IRCM; concrete (*RR: 1.56; CI: 1.09:2.22*), earth (*RR: 1.55; CI: 1.07:2.24*) and other materials (*RR: 1.82; CI: 1.15:2.90*) were associated with an increase in IRCM compared with a base material of hard core (Table 2.6).

Model 3 – Ewes housed at lambing

The base material of the floor in the barn at lambing was significantly associated with the IRCM: concrete based floors (*RR: 1.87; CI: 1.11:3.13*) were associated with an increase in the IRCM compared with a base material of hard core (Table 2.6). There was an increase in IRCM when bedding was stored covered outdoors (*RR: 2.54; CI: 1.09:5.96*) or in a building (*RR: 1.34; CI: 0.72:2.48*) compared with bedding stored outdoors uncovered (Table 2.6).

Model 4 – Ewes housed after lambing

Concrete (*RR: 2.12; CI: 1.08:4.16*), earth (*RR: 3.14; CI: 1.53:6.44*) and other (*RR: 2.16; CI: 1.05:4.43*) floor base materials were associated with an increase in IRCM compared with a base material of hard core (Table 2.6). The IRCM was lower in flocks where fresh bedding was added every 2 days or less (*RR: 0.54; CI: 0.30:0.99*), or twice a week (*RR: 0.37; CI: 0.16:0.86*) compared with flocks that had fresh bedding added weekly. Flocks that were kept in the same housing before and after lambing had an increased IRCM (*RR: 1.54; CI: 1.02:2.34*) compared with flocks that were not, but flocks that were kept in the same housing during and after lambing had a decreased risk of IRCM (*RR: 0.58; CI: 0.38:0.87*) compared with flocks that were not kept in the same housing during and after lambing (Table 2.6).

Model 5 – Ewes that lambed outdoors

Flocks with some (*RR:0.40; CI:0.22:0.73*) or all (*RR:0.55; CI:0.32:0.94*) ewes kept in the same field before, during and after lambing had a decreased IRCM, compared with those where no ewes remained in the same field before, during and after lambing (Table 2.7).

Model 6 – Ewes reared outdoors after lambing

The IRCM increased as the age of the oldest lambs at turnout increased (*RR: 1.03; CI: 1.02:1.04*) and as the mean number of lambs per ewe in the flock increased (*RR: 5.36; CI: 2.79:10.30*) (Table 2.7).

Fields that were unexposed (*RR: 14.10; CI:2.70:73.52)*, sheltered (*RR: 8.88; CI:2.52:31.24*) or a mixture of exposed, unexposed and sheltered (*RR: 26.96; CI:8.10:89.77*) had an increased IRCM compared to fields that were exposed. As the number of lambs per ewe increased in fields that were unexposed *(RR: 0.22; CI:0.08:0.60)* sheltered *(RR: 0.24; CI:0.11:0.51)* or mixed *(RR: 0.12; CI:0.06:0.25)*, the IRCM decreased (Table 2.7).

Table 2.3: Results of univariate over-dispersed Poisson regression analyses, offset by flock size, for continuous explanatory variables on farm and flock, lambing, health and feeding management associated with incidence rate of clinical mastitis.

Variable		N	RR	Lower CI	Upper CI
Percentage of flock with mastitis in previous year kept for breeding in 2009		260	1.6185	1.2618	2.0760
Percentage of flock with poor udder conformation		173	1.1311	1.0801	1.1846
Percentage of flock too fat	At tupping	293	1.0052	1.0004	1.0100
	Mid pregnancy	270	1.0095	1.0033	1.0157
	Mid lambing	285	1.0099	1.0042	1.0157
Percentage of flock with singles at scanning		128	0.9816	0.9718	0.9915
The percentage of flock with twins at scanning		129	0.9946	0.9828	1.0065
The percentage of flock with triplets at scanning		127	1.0395	1.0226	1.0566
The percentage of flock with poor udder conformation of the respondents that checked the mammary gland of ewes at lambing		247	1.0543	1.0069	1.1040
The number of lambs reared per ewe		271	0.9548	0.7438	1.2257
Percentage of lambs finished before weaning		294	1.0049	1.0018	1.0081
Percentage of flock with mastitis kept to breed		288	1.6185	1.2618	2.0760
Percentage of flock with poor udder conformation		173	1.1311	1.0801	1.1846
Age of oldest lambs at turnout (days)		233	1.0074	1.0020	1.0127

N= number of respondents who gave a valid response to the question.
RR= Risk ratio. Lower and upper CI= 96 percent confidence intervals for the risk ratio.

Table 2.4: Results of univariate over-dispersed Poisson regression analyses, offset by flock size, for categorical explanatory variables on farm and flock, lambing, health and feeding management associated with incidence rate of clinical mastitis.

Variable	Category	N	% with CM	IRCM	RR	Lower CI	Upper CI
Pedigree or commercial flock	Commercial	219	89.5	1.27		Reference	
	Both	12	83.3	1.46	1.3578	0.8760	2.1047
	Pedigree	95	78.9	1.09	0.6090	0.4769	0.7776
Texel	No	240	86.3	1.13		Reference	
	Yes	86	86.0	1.49	1.3396	1.0795	1.6624
Mule	No	173	80.3	1.17		Reference	
	Yes	153	92.8	1.29	1.4253	1.1642	1.7450
Replacement ewes	Home bred	149	81.2	1.14		Reference	
	Both	69	91.3	1.33	1.2606	0.9750	1.6298
	Bought in	106	89.6	1.28	1.4195	1.1248	1.7913
Location of ewes at lambing	Indoors	166	89.2	1.41		Reference	
	Both	81	88.9	1.25	0.7515	0.6007	0.9402
	Outdoors	78	74.4	0.82	0.4585	0.3474	0.6052
Location of ewes after lambing	Indoors	12	91.7	1.82		Reference	
	Both	176	88.1	1.41	0.9649	0.6458	1.4416
	Outdoors	130	84.6	0.99	0.6131	0.4046	0.9293
Location of ewes at all time points	Indoors	11	90.9	1.98		Reference	
	Both/changed	242	88.8	1.32	0.8575	0.5687	1.2929
	Outdoors	62	77.4	0.87	0.4213	0.2573	0.6897
Location of ewes before lambing	Indoors	230	89.6	1.39		Reference	
	Outdoors	99	76.8	0.87	1.6666	1.3160	2.1106

N= number of respondents who gave a valid response to the question. %= Percentage of flocks with CM. RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

Variable	Category	N	%	IRCM	RR	Lower CI	Upper CI
Ewes vaccinated with covexin	No	224	89.3	1.30		Reference	
	Yes	51	80.4	0.99	0.7045	0.5253	0.9450
Ewes vaccinated with scabivax	No	254	87.0	1.21		Reference	
	Yes	21	95.2	1.71	1.7629	1.2508	2.4845
Lambs vaccinated with heptavac	No	167	90.4	1.33		Reference	
	Yes	6	66.7	0.46	0.3432	0.1213	0.9712
Ewes vaccinated against pasteurella	No	54	77.8	0.99		Reference	
	Yes	221	90.0	1.31	1.3267	1.0024	1.7559
Water provisions at lambing	Restricted	274	85.4	1.31		Reference	
	Unlimited/river	27	92.6	0.76	0.5688	0.3904	0.8289
	Mix	26	80.8	0.80	0.4928	0.3468	0.7003
Water provisions after lambing	Restricted	235	86.0	1.35		Reference	
	Unlimited/river	44	84.1	0.99	0.6549	0.4774	0.8984
	Mix	48	85.4	0.81	0.8327	0.6548	1.0589
Frequency water was changed at lambing	Ad lib	30	82.2	0.76			
	Once a day	44	87.5	1.27	1.5597	1.0483	2.3206
	Twice a day	68	89.8	1.45	1.7111	1.1790	2.4833
	Three times a day	3	80.0	1.39	1.4279	0.6829	2.9858
Frequency water was changed after lambing where housed	Ad lib	45	86.7	0.79		Reference	
	Once a day	24	94.1	1.30	1.2361	0.7914	1.9305
	Twice a day	59	92.6	1.54	1.7624	1.2392	2.5066
	Three times a day	5	66.7	1.52	1.8945	0.6865	5.2279

N= number of respondents who gave a valid response to the question. %= Percentage of flocks with CM. RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

Variable	Category	N	%	IRCM	RR	Lower CI	Upper CI
How often water was topped up at lambing	Ad lib	28	92.9	0.80		Reference	
	Once a day	22	86.4	1.56	1.4862	0.9066	2.4363
	Twice a day	59	94.9	1.58	1.6923	1.1545	2.4807
	Three times a day	14	64.3	1.10	2.8226	1.7243	4.6203
How often water was topped up after lambing	Ad lib	45	84.4	0.81		Reference	
	Once a day	11	90.9	1.07	1.1658	0.6189	2.1958
	Twice a day	57	93.0	1.56	1.5951	1.0841	2.3471
	Three times a day	20	75.0	1.02	1.1041	0.6033	2.0204
Proportion of ewes that were able to eat concentrate at one time	All of the ewes	285	88.4	1.28		Reference	
	Most of the ewes	11	63.6	0.67	0.4623	0.2250	0.9497
	Less than half of the ewes	3	100.0	3.85	2.7846	1.3554	5.7207
Age at which lambs were offered creep feed	Not offered creep	180	86.1	1.15		Reference	
	Less than 1 week	14	85.7	1.60	1.0732	0.6015	1.9147
	1-3 weeks old	76	88.2	1.38	1.2835	1.0211	1.6132
	4-8 weeks old	45	80.0	1.28	1.3392	0.9905	1.8106
Proportion of ewes with mastitis treated with an anti-inflammatory	None	247	91.1	1.35		Reference	
	Some	16	100.0	1.58	1.4309	1.0327	1.9827
	All	39	97.4	1.43	1.2038	0.9200	1.5750
Base material of the floor indoors before lambing	Hard core	60	84.0	1.03		Reference	
	Concrete	110	95.7	1.69	1.5992	1.2554	2.0372
	Earth	52	87.0	1.41	1.6255	1.2373	2.1354
	Mix or other floor types	24	85.0	1.20	1.9250	1.3719	2.7009
Whether the same housing was used for ewes and lambs before and after lambing	No	122	88.5	1.25		Reference	
	Yes	104	91.3	1.57	1.2833	1.0367	1.5886

N= number of respondents who gave a valid response to the question. %= Percentage of flocks with CM. RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

Variable	Category	N	%	IRCM	RR	Lower CI	Upper CI
Base material of the floor indoors at lambing	Hard core	25	84.0	1.05		Reference	
	Concrete	125	91.2	1.52	1.6676	1.1346	2.4511
	Earth	59	86.4	1.30	1.6504	1.0790	2.5242
	Mix or other floor types	36	88.9	1.15	1.3780	0.8831	2.1501
How often fresh bedding was added to lambing pens at lambing	Daily or more	177	89.3	1.35		Reference	
	With each ewe	33	93.0	1.52	1.2282	0.9670	1.5600
	Every few days +	6	100.0	2.77	2.1201	1.2560	3.5785
	When needed	17	88.2	1.14	0.8956	0.5721	1.4020
Bedding storage	Outdoors uncovered	14	85.7	1.23		Reference	
	Outdoors covered	14	85.7	1.62	1.9921	1.0501	3.7793
	In a building	213	89.2	1.34	1.1671	0.6731	2.0236
	Mixed	5	100.0	1.73	1.2113	0.4583	3.2016
Whether the same housing was used for ewes and lambs at and after lambing	No	83	89.2	1.60		Reference	
	Yes	97	87.6	1.29	0.7569	0.5862	0.9773
How often fresh bedding was added to lambing pens after lambing	Weekly or more	7	100.0	3.00		Reference	
	Twice a week	23	87.0	1.34	0.5205	0.2833	0.9564
	Every 2 days or less	144	88.2	1.40	0.4220	0.2409	0.7392

N= number of respondents who gave a valid response to the question. %= Percentage of flocks with CM. RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

Table 2.5: Over-dispersed Poisson regression model 1, offset by flock size, of risk factors associated with the incidence rate of clinical mastitis for 148 respondents in England.

Variable		IRCM	RR	Lower CI	Upper CI
Intercept -4.1271(0.2382)			0.0161	0.0101	0.0257
Percentage of flock with poor udder conformation			1.1053	1.0505	1.1631
Number of lambs reared per ewe			0.7620	0.5439	1.0675
Management at lambing	Indoors	1.41		Reference	
	Both	1.25	0.2809	0.0888	0.8888
	Outdoors	0.82	0.0128	0.0009	0.1849
Number of lambs reared per ewe: Management at lambing	Indoors			Reference	
	Both		2.4755	1.1262	5.4413
	Outdoors		13.8925	2.1438	90.0272

RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

Table 2.6: Over-dispersed Poisson regression models 2-4, offset by flock size, of risk factors associated with the incidence rate of clinical mastitis.

Variable		IRCM	RR	Lower CI	Upper CI
Model 2 Before lambing (*n*=230)					
Intercept -4.8212(0.1773)			0.0081	0.0057	0.0114
Percentage of flock with poor udder conformation			1.1156	1.0634	1.1703
Base material of the floor	Hard core	1.03		Reference	
	Concrete	1.69	1.5575	1.0946	2.2163
	Earth	1.41	1.5508	1.0743	2.2387
	Other	1.20	1.8246	1.1492	2.8970
Model 3 At lambing (*n*=247)					
Intercept -5.1127(0.4061)			0.0060	0.0027	0.0133
Percentage of flock with poor udder conformation			1.0770	1.0050	1.1541
Base material of the floor	Hard core	1.05		Reference	
	Concrete	1.52	1.8670	1.1137	3.1298
	Earth	1.30	1.6169	0.8793	2.9733
	Other	1.15	1.5298	0.7940	2.9476
Bedding storage	Outdoors uncovered	1.23		Reference	
	Outdoors covered	1.62	2.5445	1.0857	5.9631
	In a building	1.34	1.3355	0.7181	2.4837
	Mixed	1.73	1.0289	0.2129	4.9715
Model 4 After lambing (*n*=187)					
Intercept -4.5227(0.4200)			0.0109	0.0048	0.0247
Percentage of flock with poor udder conformation			1.1372	1.0742	1.2040
Base material of the floor	Hard core	0.88		Reference	
	Concrete	1.48	2.1215	1.0824	4.1580
	Earth	1.47	3.1353	1.5260	6.4415
	Other	1.45	2.1587	1.0522	4.4287
Frequency of adding fresh bedding	Weekly	3.00		Reference	
	Twice a week	1.34	0.3675	0.1572	0.8594
	Every two days or less	1.40	0.5417	0.2970	0.9881
Ewes kept in same housing before and after lambing	No	1.25		Reference	
	Yes	1.57	1.5443	1.0199	2.3383
Ewes kept in same housing at and after lambing	No	1.60		Reference	
	Yes	1.29	0.5752	0.3789	0.8733

RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

Table 2.7: Over-dispersed Poisson regression models 5 and 6, offset by flock size, of risk factors associated with the incidence rate of clinical mastitis.

Variable		IRCM	RR	Lower CI	Upper CI
Model 5 At lambing (*n*=160)					
Intercept -4.3586(0.1638)			0.0128	0.0093	0.0176
Percentage of flock with poor udder conformation			1.0489	0.9573	1.1493
Proportion of ewes kept in the same fields before, at and after lambing	None	1.09		Reference	
	Some	0.92	0.3974	0.2175	0.7261
	All	0.90	0.5505	0.3221	0.9408
Model 6 After lambing (*n*=306)					
Intercept -7.4357(0.6063)			0.0006	0.0002	0.0019
Percentage of flock with poor udder conformation			1.0696	1.0127	1.1297
Age of oldest lambs at turnout			1.0275	1.0158	1.0394
Number of lambs reared per ewe			5.3569	2.7865	10.2986
Exposure of the fields	Exposed	1.12		Reference	
	Unexposed	1.75	14.1007	2.7045	73.5185
	Sheltered	1.14	8.8782	2.5233	31.2382
	Mixed	1.10	26.9599	8.0964	89.7726
Number of lambs reared per ewe: Exposure of the fields	Exposed			Reference	
	Unexposed		0.2171	0.0791	0.5954
	Sheltered		0.2361	0.1085	0.5139
	Mixed		0.1198	0.0567	0.2532

RR= Risk ratio. Lower and upper CI= 95 percent confidence intervals for the risk ratio. IRCM=no.ewes/100 ewes/year with clinical mastitis.

2.5. Discussion

This is the first study to estimate the IRCM and to identify management risk factors for clinical mastitis from a random sample of farmers in England.

The percentage of ewes within a flock with poor udder conformation was significantly positively associated with IRCM. This agrees with a previous longitudinal study of 67 suckler ewes, where indicators of poor conformation (pendulous udders and greater cross-sectional area of the teats) were associated with an increase in somatic cell count, indicative of subclinical infection (Huntley *et al.* 2012) and suggests that such ewes are a risk for clinical mastitis.

Flocks that were kept in the same barns during and after lambing, or in the same field before and after lambing, had a decreased IRCM. This could be because ewes' would have adapted to the microbial environment more than ewes moved to new environment for lambing: immune responses are primed by their environment.

The IRCM in flocks that were always housed was significantly higher than that in flocks that were always outdoors, and indeed any period of housing was associated with a higher IRCM. One explanation for this is that stocking density in housed ewes is higher than the stocking density of ewes at pasture. There is an increased bacterial load with increased stocking density (Sevi *et al.* 1999). In the housed environment bacterial contamination would be exacerbated by contaminated straw and in the current study bedding stored covered outdoors, where it might become warm and damp and bacteria could grow was associated with higher IRCM. Contact with bacteria can be reduced by a layer of fresh straw and in the current study a higher rate of addition of fresh straw was associated with a lower IRCM. Deep straw bedding at calving was negatively correlated with IRCM in a study of 274 dairy cow herds (Barkema *et al.* 1999).

In the current study, concrete, earth and other materials were associated with an increase in IRCM compared with hard core. Several studies of other ruminants have identified associations between floor type and mastitis. A longitudinal study of 315 dairy goats found an increase in the detection of bacteria in milk of does housed on earth floors compared with does housed on raised timber floors (Ndegwa *et al.* 2000). In a study of 245 dairy cows in southern Ethiopia, cows in houses with soil

floors had a higher IRCM than cows on concrete floors (Abera *et al.* 2012). In a cross-sectional study of 1923 dairy farms by Ruud *et al.*, 2010, the IRCM decreased on flooring materials such as rubber, multilayer mats and mattresses compared with concrete (Ruud *et al.* 2010). The association between floor type and the infection status of an animal is probably linked to the how easy it is for a flooring material to be cleaned. In this study hard core flooring resulted in a decrease in IRCM compared to other floor types. This seems surprising but could be because hard core, being more uneven compared to the other floor types, allows for better drainage of fluids reducing the likelihood of bedding becoming moist, which would aid bacterial colonisation. Alternatively it could be that bedding depth is greater to protect ewes from the hard core. This finding warrants further investigation.

The IRCM in a flock increased as the age of the oldest lambs at turnout increased. This could be used as a proxy for the length of time ewes and lambs were kept indoors after lambing; the longer ewes and lambs were kept indoors, the higher the risk of clinical mastitis. This may be due to build-up of bacterial contamination.

Farmers were asked to describe fields as exposed, unexposed or sheltered. Flocks with unexposed, sheltered or mixed fields were at increased risk of clinical mastitis compared to those with exposed fields. Ewes and lambs in fields with areas that are unexposed or have shelter are likely to cluster in these areas, resulting in wet, muddy areas that may act as reservoirs for mastitis causing pathogens and increase transmission from ewe to ewe.

Several studies have shown ewes rearing several lambs are more susceptible to mastitis than ewes rearing a single lamb (Arsenault *et al.* 2008, Gross *et al.* 1978, Larsgard & Vaabenoe 1993, Waage & Vatn 2008, Watkins *et al.* 1991). In this study, the number of lambs reared per ewe was only significant when an interaction with management practice was taken into account. This difference is likely to be due to respondents that managed ewes indoors having different management practices for ewes with twins or triplets, for example ensuring supplementary feed and checking on these ewes more regularly. Ewes with more than one lamb that are outdoors will have less assistance then those lambed indoors and will have increased cumulative stress on the mammary gland which might result in intramammary infection.

There are many challenges to postal questionnaires, including respondent bias, under-estimation of disease prevalence, validation, ensuring respondents understand questions and low response rates to both the questionnaire as a whole and individual questions which all could alter the results of the study.

Previous studies have also identified breed to be a risk factor for mastitis (Larsgard & Vaabenoe 1993). In this study, this factor was also significant during univariable but dropped during multivariable analyses. Other studies have reported that ewe age, geographical region and ewe body condition were risk factors for mastitis (Arsenault *et al.* 2008, Waage & Vatn 2008, Watkins *et al.* 1991). In this study we did not find any of these risks were significantly associated with IRCM during univariable analyses. In the case of geographical region, this could be because the previous studies were undertaken outside of the UK where the environment (such as weather conditions, vegetation, topology and soil composition) may vary more between each geographical region, unlike the UK where these variations are less extreme.

Only 87.2% of respondents checked ewes' mammary glands at lambing, and the regularity of these checks reduced throughout lactation. Teat lesions are relatively transient in nature compared to udder conformation traits that are less changeable overtime. The irregularity of checks coupled with brief teat lesion cases might have resulted in an under-estimation of teat lesions, and therefore an inaccurate identification of the association between them and IRCM.

Ewe age was also not significantly associated with IRCM unlike many studies. This was likely to be because many respondents gave invalid responses for questions on ewe age, often omitting the number of ewes within each age group therefore resulting in inaccurate results.

The design of some questions may have resulted in associations between explanatory variables and the IRCM being missed. For example in this study, the categories available for body condition of ewes were 'too thin', 'about right' or 'too fat'. These categories are subjective and would have varied by farm. The categories gave us no information regarding weights or body condition that might be associated with the IRCM, particularly as most respondents thought their ewes were 'about right'.

Postal questionnaires depend on the respondents' willingness to participate. Selective non-response might lead to bias in the prevalence of disease (Hardie *et al.* 2003, Hoeymans *et al.* 1998). In this study, respondents may have had a different IRCM than non-respondents resulting in an under or over-estimation in IRCM.

Lack of responses to individual questions means that variables cannot be analysed resulting in 'missed' risk factors. For example, nutrition is associated with the IRCM in dairy herds (Barkema *et al.* 1999). In this study, although nutrition was investigated, there were low response rates to these questions, and therefore they could not be included in the Poisson regression models. Thirty nine respondents did not provide the number of ewes with CM and/or the number of ewes in the breeding flock which immediately resulted in them being excluded from further analysis. These respondents may have varying IRCM, thereby resulting in an inaccurate IRCM.

Under or over-estimation of values is a drawback of postal questionnaires where there is no validation. Validation of a postal survey could involve a visit to the study site, or a further interview with the respondent and might show respondents have not reported accurate values on the survey. Only 87.2% of respondents checked ewes' udders at lambing. The remaining 12.8% might be under-estimating cases of clinical mastitis. Even those that do check ewes' udders may not be specifically looking for signs of mastitis, or can recognise clinical mastitis. In the current study, the regularity of these checks decreased from the first week after lambing to 5-8 weeks in lactation with less than 50.0% of respondents checking ewes every day in the first week. Cases may be missed or misclassified as acute clinical rather than chronic cases due to irregularity of checks, and these cases are more likely to be missed or misclassified as time goes on.

There are many explanations for under-estimating the IRCM in this study. The IRCM estimate in this study of 0.8 and 1.9/ 100 ewes / year for outdoor and indoor flocks respectively should be considered to be the bottom limit of this estimate in England. The prevalence of subclinical mastitis is likely to be substantially higher than this as there are no clinical signs of the disease and cases will be missed more frequently.

This was a hypothesis generating study, and variables need to be tested further in longitudinal studies to confirm statistical associations as well as the direction of these associations. The ewes' microbial environment and host susceptibility (as indicated by udder conformation) were highlighted as potential avenues to investigate.

2.6. Conclusions

This cross-sectional study provided an estimate (1.2 / 100 ewes / year) for the incidence rate of clinical mastitis in sheep in England. Possible risk factors for clinical mastitis in England were identified including udder conformation, and indoor and outdoor management practices before, at and after lambing. These risk factors could be investigated in more detail in longitudinal studies and clinical trials in order to assess causality.

Chapter 3. Identification of environmental reservoirs of bacteria that could cause intramammary infections using MALDI-ToF-MS

3.1. Introduction

Colonisation by a potential pathogen is the first step in infection so the identification of a bacterial reservoir can give an indication of a possible transmission pathway in a disease process. Improved knowledge of bacterial species associated with mastitis, including reservoirs and transmission routes would form a better basis for implementing control measures.

Different strains within a bacterial species can vary in terms of reservoir, transmission pathway, persistence and pathogenicity. Strain typing is therefore necessary to identify sources and transmission routes of mastitis-causing bacteria and to choose appropriate prevention and treatment measures. Although contributing to our understanding of mastitis, molecular epidemiological studies have not yet reached a stage where strain typing is time and cost effective enough to be used in real time to contribute to outbreak analysis and prevention and/or treatment options. Ideally, a method of strain typing that is rapid and cost effective is required in order for large numbers of samples from numerous animals to be analysed. MALDI-ToF-MS, due to its speed and low running costs, has the potential to replace conventional phenotypic identification. In addition, several recent studies have indicated MALDI-ToF-MS could be used for rapid strain differentiation and identification for *Escherichia coli* (Christner *et al.* 2014, Karger *et al.* 2011), *Yersinia enterocolitica* (Stephan *et al.* 2011), *Salmonella enterica* (Dieckmann & Malorny 2011), *Clostridium difficile* (Reil *et al.* 2011), *Staphylococcus aureus* (Boggs *et al.* 2012, Wolters *et al.* 2011), *Propionibacterium acnes* (Nagy *et al.* 2013), *Listeria* species (Barbuddhe *et al.* 2008), *Leptospira spp.* (Rettinger *et al.* 2012), *Saccharomyces cerevisiae* (Moothoo-Padayachie *et al.* 2013), Mycobacteria species (Hettick *et al.* 2006), and *Arthrobacter* species (Vargha *et al.* 2006).

There are three routes for bacteria to invade the mammary gland and cause infection: galactogenic route of entry (that is through the teat canal, with ascending infection),

haemotogenous spread which would diffuse through mammary paranchyma, and percutaneous entry (wounds) (Meador 1988). There are also several potential reservoirs of bacteria, such as the lamb's mouth, the ewe's udder skin and/or the environment, such as the bedding (Gougoulis *et al.* 2008b, Piccinini *et al.* 2009). The infected udder gland is also a reservoir for transmission between individual udder halves (Mørk *et al.* 2007, Vautor *et al.* 2003) and between ewes.

Although there has been increased interest in reservoirs and transmission routes of pathogens associated with bovine mastitis, particularly in dairy cattle, little is known about the reservoirs and transmission pathways associated with suckler ewes. At the species level, *Mannheimia haemolytica* has been identified in the mouths of ewes and lambs, and on the teat skin of pregnant and lactating ewes which may enable it to invade the mammary gland during suckling (Fragkou *et al.* 2011, Scott & Jones 1998). In a study of 11 ewes, *Mannheimia haemolytica* was isolated from the teat duct after suckling only, indicating it had been transmitted to the teat duct during suckling by the lamb (Gougoulis *et al.* 2008b). Although these studies provide accumulating evidence for reservoirs and transmission routes of pathogens associated with intramammary infections, molecular methods at the subspecies level are required in order to ascertain whether the isolates are the same strain type, therefore proving a transmission event has occurred. A single study on suckler ewes has identified one reservoir and transmission route of mastitis causing bacteria where frequent transmission of *Staphylococcus aureus* was found between the dam and her lambs using pulse-field gel electrophoresis - the current gold standard for strain differentiation for staphylococci (Mørk *et al.* 2012b).

This study therefore aims to investigate ewe udder skin as a reservoir for bacteria that enter the mammary gland, and to assess the use of MALDI-ToF-MS as a means by which to complete this aim.

3.2. Aims and objectives

The aim of this study was to identify environmental reservoirs of bacteria that might be associated with intramammary infections in sheep using Matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF-MS). This was achieved through:

1. Assessing the use of MALDI-ToF-MS as a strain clustering method for bacterial isolates from ewe udder skin and milk by comparison to an optimised pulsed-field gel electrophoresis (PFGE) method.

2. Identifying paired strain types from ewe udder skin and milk that indicate that udder skin is an environmental reservoir for bacteria found in the milk.

3.3. Materials and methods

3.3.1. Flock and animals

A flock of approximately 100 pedigree Texel suckler ewes was selected. Twenty-eight milk samples from 27 ewes with clinical mastitis were collected by a veterinarian in August 2010. In October 2010, a further 5 milk samples were taken from 3 ewes with no signs of clinical mastitis and 14 udder skin swabs were collected from 10 ewes (8 from 7 clinically infected ewes and 6 from 3 clinically uninfected ewes).

3.3.2. Sampling

Milk

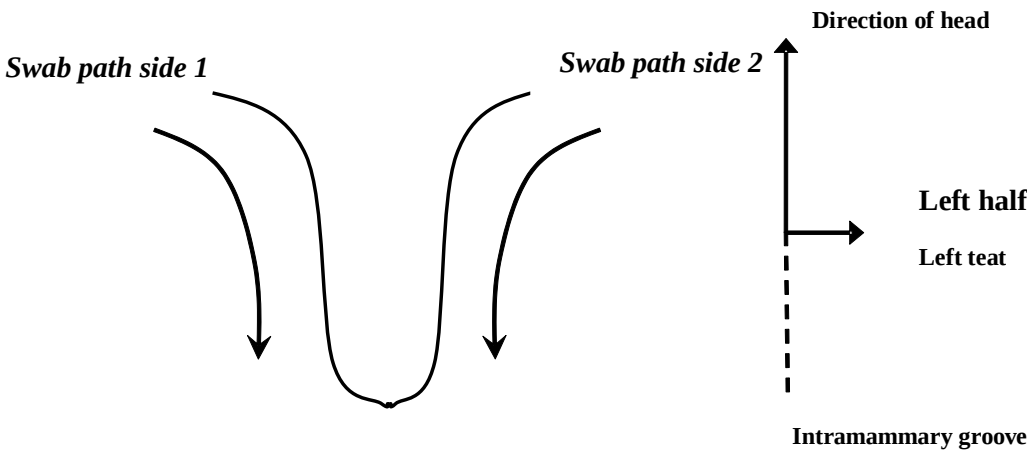
Milk samples (approx 5 ml) were taken in August 2010 by a veterinarian into a sterile universal and transported to the University of Warwick on ice. Milk samples were taken from the affected udder half in ewes with clinical mastitis. In one case, both udder halves were affected and so two milk samples were taken. For those milk samples collected in October 2010, the teats were disinfected with 70% ethanol wipes and the first streams of milk discarded. Udder half milk samples (approx 5 ml) were aseptically collected into pre-labelled universal tubes and stored with glycerol

(10% v/v). In 2 of the 3 ewes without signs of clinical mastitis, milk samples from both halves were taken.

Udder skin swabs

The ewe to be tested was turned over and swabs were placed in 1 ml of Phosphate buffered saline (PBS) (to facilitate collection of viable bacteria from the dry surface of the teat) and then run along the outside of the teat base to tip, twisted and then run along the inside of the teat (Figure 3.1). The tip of the teat was not tested as this was the area held to keep the teat steady. Swabs were immediately broken off into 1 ml of Brain Heart Infusion media (BHI) (3.7% w/v Sigma-Aldrich) and glycerol (10% v/v) into a cryovial tube. Udder skin samples were taken from both halves.

Figure 3.1: The technique used to take a teat skin sample with a swab.



3.3.3. Swab and milk sample storage

Milk (with glycerol added to 10% v/v) and skin swab samples (in BHI and 10% v/v glycerol) were stored and transported on ice to the laboratory (University of Warwick). They were then stored at -20°C until analysis.

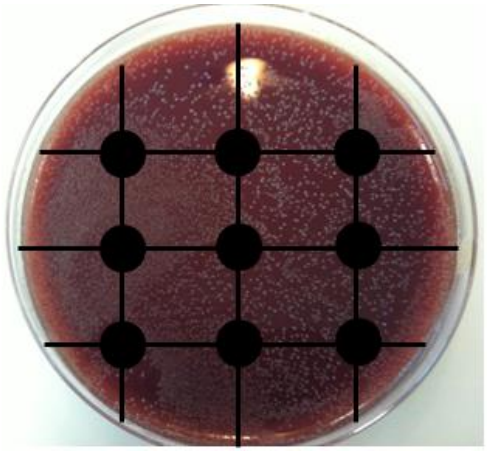
3.3.4. Growth and selection of isolates from agar plates

Milk

In July 2011, milk samples were thawed at room temperature and 100µl of milk were spread across a sheep's blood agar (SBA) plate (3.7% w/v BHI and 1.5% w/v Bacto Agar (Sigma-Aldrich 2014)) containing 5% (v/v) sterile defibrinated sheep's blood (Oxoid 2014c). Plates were incubated inverted at 37°C and checked at 24 and 48 hours.

At least one of each morphologically unique isolate was selected for further analysis. For plates with heavy growth (>30% of the plate was covered in bacterial growth) of morphologically identical isolates, up to 9 of each morphologically unique isolate were picked from 9 locations on the plate where 3 gridlines vertically and horizontally met (Figure 3.2).

Figure 3.2: Location of isolates from milk picked where there was heavy growth.

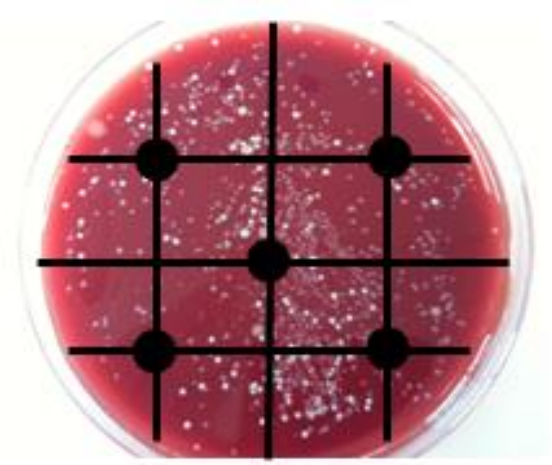


Udder skin swabs

In July 2011, udder skin swab samples in 1 ml of BHI and glycerol (10% v/v) were thawed at room temperature. Once thawed, the cryovial tube was mixed gently by pipetting and 100µl was spread across a sheep's blood agar (SBA) plate containing 5% (v/v) sterile sheep's blood. Plates were incubated inverted at 37°C and checked at 24 and 48 hours. At 24 hours (and 48 hours if new colonies had grown between this time), individual colony types were selected for further analysis.

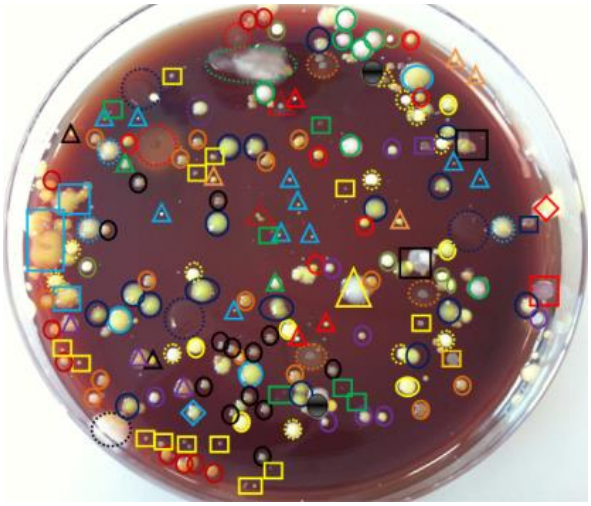
At least one of each morphologically unique isolate was selected for further analysis. For plates with heavy growth of morphologically identical isolates, up to 5 of each morphologically different isolate were picked from 5 locations on the plate (Figure 3.3).

Figure 3.3: Location of isolates picked from udder skin swabs where there was heavy growth.



For plates with >3 of a variety of morphologically unique isolates (example shown in Figure 3.4), plates were photographed and annotated in Microsoft Word, with morphologically identical isolates highlighted with the same colour and shape (Figure 3.4). Isolates of all distinct morphological types were selected after plate annotation.

Figure 3.4: Example of annotation of morphologically similar isolates.



Contrary to dairy industry standards, which assume milk sample plates with >3 morphologically distinct colonies are contaminated; there was no number of distinct colonies types that were defined as "contamination". This was particularly relevant to the highly diverse skin swabs.

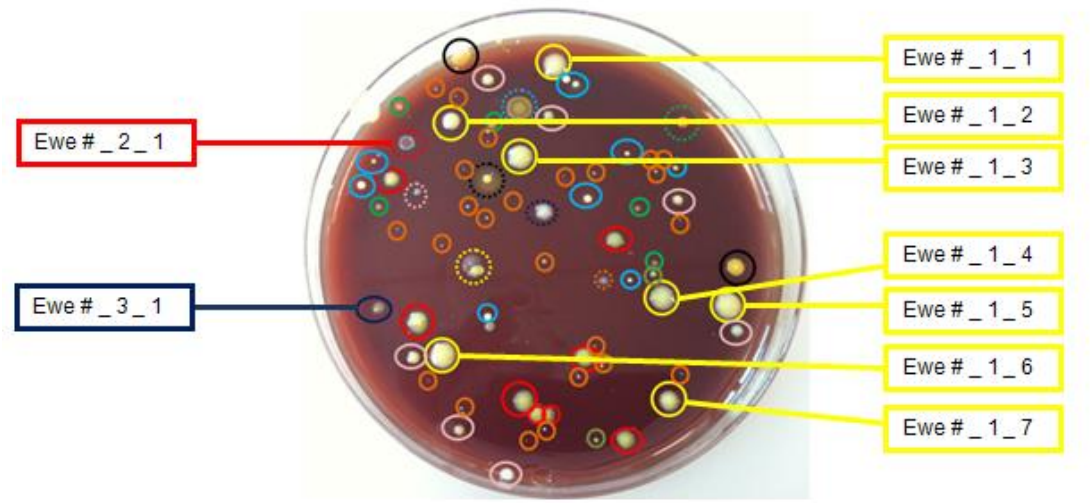
3.3.5. Isolate culturing and storage

Once selected, individual colony types from milk and udder skin samples were sterile streaked using a sterile loop on a brain heart infusion plate (BHI) and incubated inverted at 37°C and checked at 24 and 48 hours. A single colony was then picked and sub-cultured onto one quadrant of a BHI plate to provide sufficient culture for further testing (Figure 3.5).

Figure 3.5: Workflow from sample plate, to sterile streaking to quarter plating.

Plates were labelled with ewe name, udder half (if applicable) and sample type (udder skin swab or milk). For a single plate, isolates were identified by a system highlighted in Figure 3.6. Therefore, **ewe** = the ewe number, morphologically unique **isolate** = the second number in the series, which would indicate a morphologically unique isolate and **colony** = isolates with the same morphology but which had separate colonies.

Figure 3.6: Example of the annotation and labelling of a single milk or skin sample from one ewe.



A 1µl loop of growth from each quadrant was placed in 5 ml of BHI and glycerol (10% v/v) in a glass universal and incubated at 37°C with vigorous shaking at 200 revolutions per minute (rpm) until turgid (usually at least 24 hours). A 1 ml aliquot of the incubated culture was transferred into a cryovial tube, labelled, snap frozen in dry ice and stored at -80°C.

3.3.6. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis of isolates

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) allows the analysis of biomolecules by vaporizing protein molecules with laser energy, yielding distinct signals (mass spectra) for each sample which can be compared to a database to identify bacteria to the species level.

Fresh growth of isolates was prepared prior to MALDI-ToF-MS analysis at QMMS, Somerset.

Sample preparation

Direct transfer

A cocktail stick was dabbed into a distinct single colony and zig-zagged across two sequential spots on a Bruker 96 well sample plate. Matrix (1µl) was placed on top of each sample. Matrix was made up using 1 tube of Bruker "HCCA matrix portioned"

(Bruker Daltonics) and 250 µl of an "organic solvent" (50% acetonitrile (AN), 2.5% trifluoracetic acid (TFA) and 47.5% deionized H_2O). The prepared matrix was stored in the dark at room temperature and used within 2 weeks.

The bacterial test standard (BTS) (Bruker Daltonics) was used as a control. BTS was made up using a Bruker BTS portioned sample, and 50µl of organic solvent. The direct transfer method was used initially for each isolate.

Extraction method

For some isolates, analysis using the direct transfer method did not produce suitable spectra (usually characterised by a high background and low peak intensity). In these cases the extraction method was used to prepare a sample for MALDI-ToF-MS analysis and so obtain a clearer spectrum. 300 µl of distilled H_2O was aliquoted into an Eppendorf tube. A single colony was picked from a quadrant streaked plate using a 1 µl sterile loop and placed into the tube. 900 µl of ethanol was added and mixed thoroughly. This solution as then centrifuged at 13,000 rpm for 4 mins and the supernatant discarded. This was repeated to remove residual Ethanol and the pellet was left to air-dry for up to 30 minutes. 50 µl of 70% formic acid and 50 µl of pure acetonitrile was added to the pellet in a fume hood and pipette mixed. This was centrifuged at 13,000 rpm for 2 mins. The supernatant (1µl) was placed onto the target plate in duplicate and allowed to air dry. This was overlaid with 1 µl of the matrix solution and air dried.

Species identification

Sample plates were then analysed using MALDI-ToF-MS following the manufacturers instructions. The spectra produced by MALDI-ToF-MS were automatically compared to every spectra in the Bruker database. A species name for each isolate and a 'confidence score' were assigned to each isolate. The score value for each isolate was automatically calculated by MALDI Biotyper 3.0 (utilising Matlab), using a matching algorithm that computes different characteristics of two spectra to compare patterns within the spectra. Between two spectra, the number of signals that have a closely matching partner, and the symmetry of the matching signal pairs are determined. If the number of signals and the intensity of signals of each spectrum correspond, this results in a high matching value. Bruker Daltonics

suggests a conservative score of 2.0+ to be used for species identification. In this study, a score of 1.7+ was used as a cut-off point due to the additional confirmation of species identification through the number of technical repeats, culture morphology, Gram staining results and use of Pulsed-field gel electrophoresis.

The results were saved in HTML format and transferred into Microsoft Access. Queries were used to merge technical repeats, allowing the top score for each colony to be identified. Isolates with at least one score of ≥1.7 were included in analysis. The species level identification was also compared to previous laboratory analyses (culture morphology, and Gram stain) to ensure accuracy.

Strain differentiation

Data analyses were carried out in MALDI Biotyper software 3.0, ClinProTools, and Flex Analysis. Several parameters for dendrogram and correlation coefficient indices (CCI) production in MALDI Biotyper 3.0 were tested, and the literature was reviewed. Dendrograms (Distance measure: correlation, Linkage: average) were used to cluster isolates into groups and CCI estimates (Mass 3000-12000, Resolution: 4) were used to compare isolates of interest. Ultimately only CCI images were used as values were absolute, whereas dendrogram distances changed depending on the isolates inputted into the analysis. CCI matrices were colour coded according to Microsoft Excel conditional formatting to easily view isolates that were very similar (high CCI estimates in red) or very different (low CCI estimates in green).

Isolates from species found in abundance on both udder skin and in milk were taken forward for further analysis (Pulsed-field gel electrophoresis) including isolates identified as *Staphylococcus equorum, Staphylococcus warneri, Rhodococcus corprophilus, Micrococcus luteus,* and *Bacillus pumilus.*

3.3.7. Pulsed-field gel electrophoresis

The typing method is considered the gold standard for analysing a number of organisms. The protocol was based on McDougall *et al.,* (2003). In brief, cells grown to a specific optical density were immobilised in low melting point agarose gel plugs and the immobilised cells were enzymatically lysed within the gel matrix. Cell debris was then washed from the plugs leaving behind essentially intact DNA

molecules in the plug. The method was specifically optimised for all species found on both udder skin and in milk to enable high quality PFGE analysis in future studies.

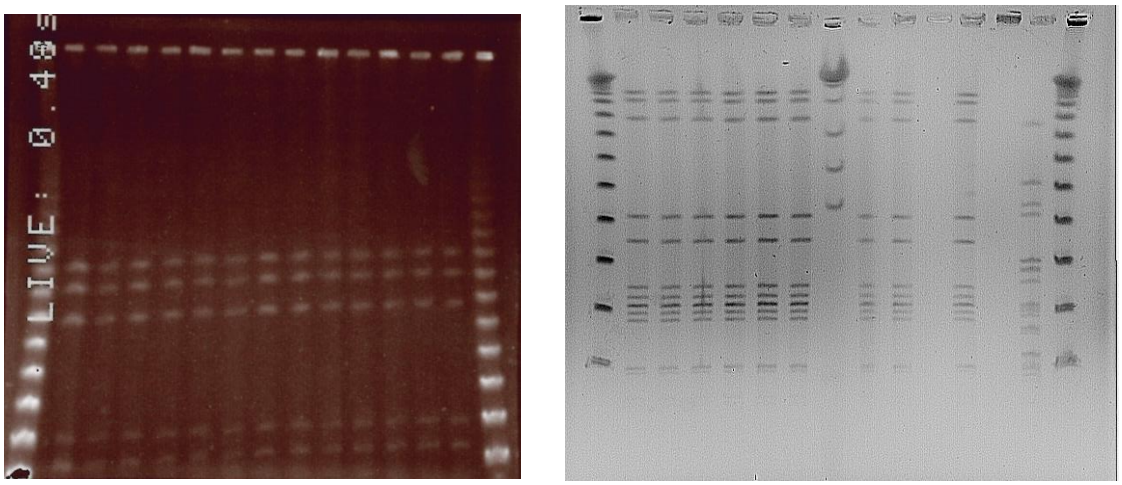
Several parts of the method, based on McDougall *et al.,* 2003, were optimised in order to improve PFGE gel quality and subsequent analysis (Table 3.1). An example of the original versus optimised gel image show tighter bands, suitable run time, and optimal image acquisition is shown (Figure 3.7).

Table 3.1: Optimisation parameters compared to original method McDougal *et al.*, 2003 and the improvements these optimisations had.

PFGE stage	PFGE Parameter		Original	Final	Reason
Plug production	Growth media		5 ml BHI	5 ml BHI and glycerol (10% v/v)	Improved growth of all isolates
	Cell concentration		Abs 0.9-1.1 (OD_{610})	Abs 0.4-1.1 (OD_{610})	Altered depending on genus, as too high an OD meant too many cells were in the plugs which hindered lysis, or too low an OD meant bands would not be visible
	Plug agarose concentration		1.8%	1.2%	Improved final gel image (possibly due to lysis and restriction digest being better with a less concentrated agarose)
	Equilibration of culture and agarose		10 mins at 37°C	25 mins at 50°C	Improved lysis, and ensured cell suspension was at a similar temperature to agarose improving mixing and encouraging uniform plugs.
Lysis	Addition to 300µl cell suspension	Proteinase K (20mg/ml)	None added	25µl (all species apart from staphylococci)	Improved lysis and therefore final gel image for all but staphylococcal species.
		Sodium dodecyl sulfate (SDS) (10% w/v)	None added	25µl (all species apart from staphylococci)	Improved lysis and therefore final gel image for all but staphylococcal species.
		Lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate)	4µl	3µl (staphylococcal spp. only)	3µl was a suitable amount to ensure cell lysis
		Lysozyme (20mg/ml)	NA	20µl (all species apart from staphylococci)	Improved lysis and therefore final gel image
	Addition to 3ml lysis buffer	Proteinase K (20mg/ml) and Lysostaphin stock solution (1 mg/ml in 20 mM sodium acetate)	None added	Added	Addition of proteinase K and lysozyme in the lysis buffer further improved lysis.
	Lysis incubation time		4 hr	Overnight	Improved lysis

PFGE stage	PFGE Parameter	Original	Final	Reason
Wash	Number of washes	4	6	Additional washes were required for ensure Proteinase K and SDS were removed
	Composition	TE only	TE X 4, ultrapure sterile water x 2	Ultrapure sterile water washes reduced the smearing of gel images.
Restriction digestion	BSA	NA	1%	Improved restriction digest substantially reducing time needed to achieve complete digestion
	Digestion temp	25°C	30°C for staphylococcal species, 37°C for all others	Allowed complete restriction digest
	Digestion incubation length	2-3hr	Overnight for staphylococcal species. 4 hours for all other species.	Allowed complete restriction digest
Running parameters	Gel concentration	1%	1.2%	Encouraged clear band separation (especially for bands that were extremely close) and reduced smearing.

Figure 3.7: Original versus optimised Pulsed-Field Gel Electrophoresis gels



Original Optimised

Final PFGE method used

One colony was transferred from a quadrant streaked plate to 5 ml BHI and glycerol (10% v/v) liquid broth in a glass universal. This was incubated for varying times and temperatures in a shaking incubator (170 rpm) depending on the genus of the isolate (Table 3.2).

Table 3.2: Incubation time and temperature by genus.

Genus	Temperature	Time
Bacillus	25°C	24 hours
Micrococcus	25°C	24 hours
Rhodococcus	25°C	48 hours
Staphylococcus	37°C	24 hours

Plug production

200 µl of liquid culture was transferred to a fresh glass universal containing 5ml BHI and glycerol (10% v/v) for the same time, at the same temperature. The OD_{610} of the cell suspension was adjusted with PBS (Table 3.3).

Table 3.3: Final concentration of cell suspension range by genus.

Bacterial genus	Concentration of cell suspension @610nm
Bacillus	0.800-1.000
Micrococcus	0.800-1.000
Rhodococcus	0.400-0.700
Staphylococcus	0.900-1.100

The cell suspension (200 µl) was centrifuged at 12,000 rpm for 3 minutes. The supernatant was aspirated and the pellet resuspended in 300µl of 1 ml Tris-EDTA (TE) buffer (10 mM Tris HCl, 1 mM EDTA [pH 8]).

Preparation of bacterial cell agarose plugs and subsequent cell lysis

Staphylococcal species: 4µl of conventional lysostaphin stock solution and 296µl of molten 1.2% SeaKemGold agarose in TE buffer [pH 8] were added to the cell suspension, gently mixed and dispensed into the wells of a plug mould.

All other species: 20µl of lysozyme (20mg/ml), 3µl of lyzosyme (1mg/ml), 25µl of 10% Sodium dodecyl sulfate (SDS), and 25µl of Proteinase K (20mg/ml) were added to the cell suspension and incubated at 50°C for 25 minutes. 227µl of 1.2% SeaKemGold agarose in TE buffer [pH 8] were added to the incubated cell suspension and gently mixed, and dispensed into the wells of a plug mould.

Plugs were left to solidify at room temperature for 10-15 minutes and then removed from the plug molds and placed in a sterile plastic bijoux containing 3ml of lysis buffer (6 mM Tris HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij-58, 0.2% sodium deoxycholate, 0.5% sodium lauroylsarcosine, 0.83mg/ml Proteinase K, 0.83mg/ml lysozyme). The tubes were then incubated overnight at 37°C in a shaking incubator (190 rpm). The plugs were transferred to a sterile plastic bijoux containing 3ml of TE wash and incubated in a shaking incubator at 37°C for an hour. This was repeated four times. The plugs were washed in the same manner with ultrapure sterile water twice. Plugs were stored in fresh TE at 4°C.

Restriction digestion of extracted DNA in agarose plugs

A whole plug slice was then transferred to a sterile eppendorf containing the restriction enzyme, restriction buffer and BSA for varying lengths of time depending on the genus, and restriction enzyme (Table 3.4).

Gel preparation and agarose plug loading

In order to prepare the gel, SeaKemGold agarose was prepared in 0.5X TBE (1.2%) and poured into the gel mold, which was left to solidify for an hour. The comb was removed and plug slices were washed in ultrapure sterile water, dried and loaded into wells.

PFGE running parameters

PFGE was performed using contour-clamped homogeneous electric field (CHEF) apparatus (CHEF Mapper, Bio-Rad, USA). Running parameters were as follows: 200 V (6 V/cm); temperature, 14°C; initial switch, 5 s; final switch, 40 s; which changed over the entire run and time, 21 h. Gels were stained in 0.5mg/ml ethidium bromide solution for 30 minutes in a covered container and then destained in distilled water for 1 hour. The water was changed twice during this time. Gels were viewed by UV transillumination and the images stored for further analysis.

Table 3.4: Restriction digest enzymes, amounts, temperatures and lengths by genus.

Bacterial genus	Enzyme	Amount (µl)				Temperature (°C)	Time (hours)
		Restriction buffer 10X	H_2O	BSA (1%)	Restriction enzyme		
Staphylococcus	SmaI	20	180	6	3	30	18
Rhodococcus	AseI	20	180	5	5	37	4
Bacillus	ApaI	20	180	5	5	37	4
Micrococcus	XbaI	20	180	5	4	37	4

3.3.8. Data management

GelCompar II (Applied Maths, Belgium) was used to cluster isolates. Gel images were imported into the software, and edited to select lanes, bands, curves and to normalise between gels using ladders on either side of the gel. Clustering (Band based: different bands, Dendrogram type: UPGMA (unweighted pair group mean average)) was carried out to cluster isolates of the same or different PFGE types. Isolates were considered to be different strain types if they had ≥1 band difference (dendrograms did not have 100% similarity for those isolates). This is because isolates that are indistinguishable by PFGE are likely to have been derived from a common parent (Tenover *et al.* 1995), which is necessary in terms of outbreak, reservoir and transmission analyses.

Dendrograms produced from GelCompar II were compared to CCI indices from MALDI Biotyper 3.0 to assess the use of MALDI-ToF-MS as a strain clustering method. PFGE images in GelCompar II were also used to identify paired strain types in milk and on udder skin. Conditional formatting in Microsoft Excel was used to produce a colour scale for each CCI matrix.

3.4. Results

3.4.1. Descriptive analysis

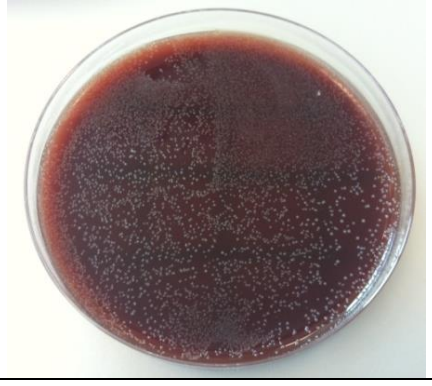
A summary of the number of ewes, samples, number of unique and total isolates with and without a sufficient ID (one of the replicates with a score of ≥1.7) from MALDI-ToF-MS is shown in Table 3.5. The majority (85.9%) of isolates from milk from all ewes, whether with or without clinical signs of mastitis, were given a sufficient ID by MALDI-ToF-MS. The percent of isolates from swab samples that were given a sufficient ID was substantially lower (51.9%). Despite this, very few isolates from all three sample types (milk, udder skin swabs and teat skin swabs) had no spectra produced, and therefore with future database updates, these isolates may become identifiable in the future.

Table 3.5: A summary of the number of ewes, samples, number of unique and total isolates with and without a sufficient ID from MALDI-ToF-MS.

	Summary	Milk from ewes without CM	Milk from ewes with CM	Swabs
Ewes		3	27	10
Samples	Total number	5	28	14
	Number with growth	5	25	14
Morphologically unique isolates	Total number	10	97	368
	Number with a sufficient ID (≥1.7)	10	84	177
	Number with an insufficient ID (<1.7)	0	13	191
Total isolates	Total number	70	270	412
	Number with a sufficient ID (≥1.7)	62	230	214
	Number with an insufficient ID (<1.7) with spectra	7	35	190
	Number with an insufficient ID (<1.7) with no spectra	1	5	8

The species and photographs of the plates for each udder half of each ewe without clinical mastitis are shown in Figure 3.8. Interestingly, the milk samples from ewes without any clinical signs had heavy growth of one or more bacterial species. The left and right udder halves did not necessarily have the same species present. Growth from these milk samples included species typically associated with mastitis.

Figure 3.8: The growth and bacterial species present (as identified by MALDI-ToF-MS) for milk samples from ewes with no clinical signs of mastitis.

Ewe number	Left sample	Right sample
106	*Staphylococcus simulans* 	*Escherichia coli* 
37	*Staphylococcus simulans* *Streptococcus uberis* *Morganella morganii sibonii* 	*Staphylococcus chromogenes* *Staphylococcus simulans* 
32	*Streptococcus uberis* *Staphylococcus chromogenes* *Staphylococcus haemolyticus* 	NA

The percentage of ewes with clinical mastitis by the most frequently isolated bacterial species is shown in Figure 3.9 for mastitic milk, and Figure 3.10 for udder skin. A wide range of bacterial species were isolated from the 27 ewes with clinical mastitis. *Arthrobacter gandavensis*, *Bacillus pumilus*, and *Micrococcus luteus* were the most frequently isolated species from both udder skin and mastitic milk. *Staphylococcus* species were more frequently isolated from mastitic milk rather than udder skin. Interestingly, *Escherichia coli*, *Mannheimia haemolytica* and *Streptococcus uberis*, species typically associated with mastitis, were only isolated from ewes without clinical signs of mastitis.

Figure 3.11 shows the percentage of ewes from which bacterial species were isolated from both mastitic milk and udder skin samples. These isolates were of particular interest as, if they were the same strains, could indicate that the udder skin is a reservoir of infection.

Figure 3.9: Percentage of ewes with each species of bacteria present in milk (from ewes with clinical mastitis).

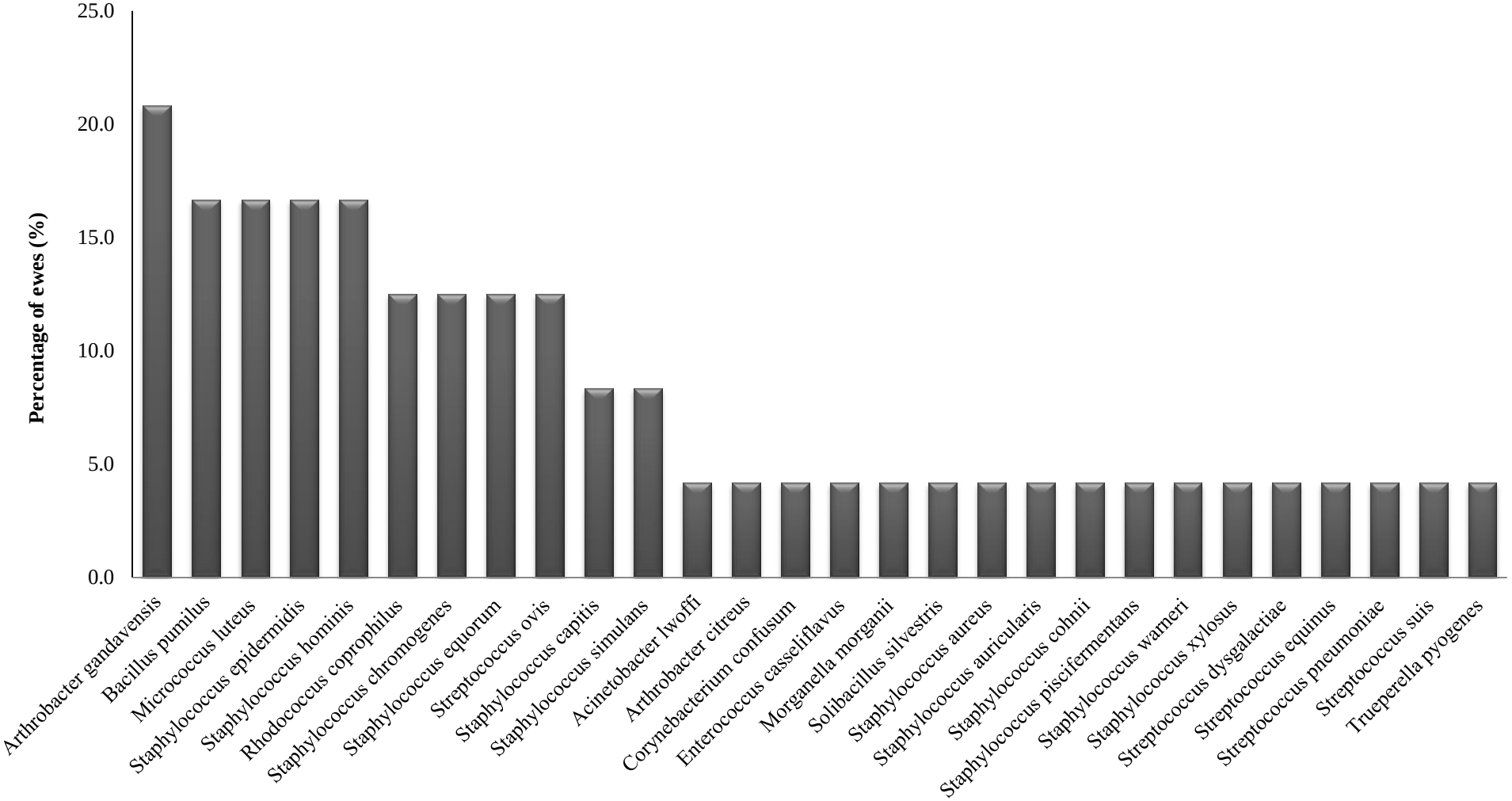


Figure 3.10: Percentage of ewes with each species of bacteria present on udder skin.

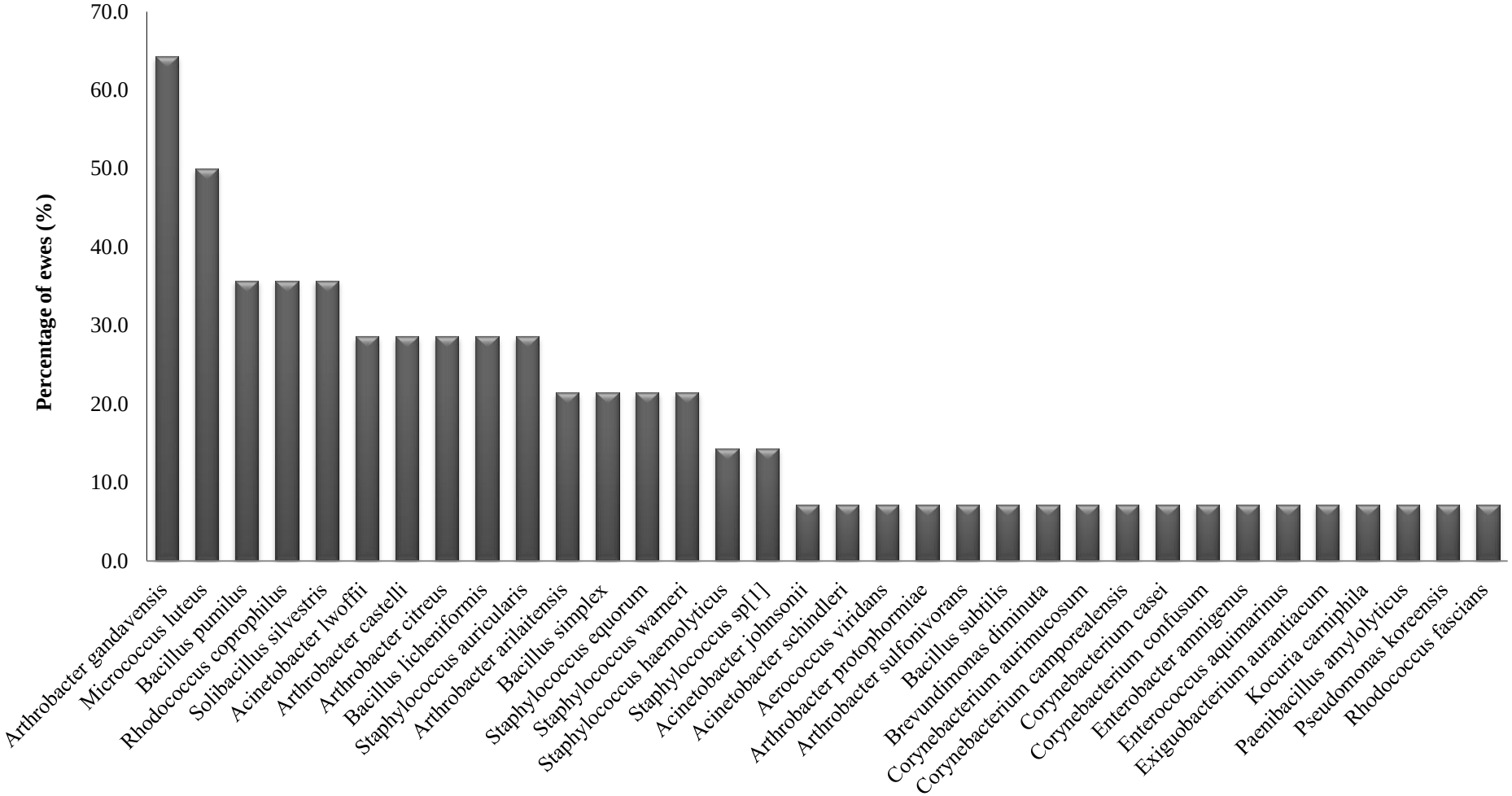
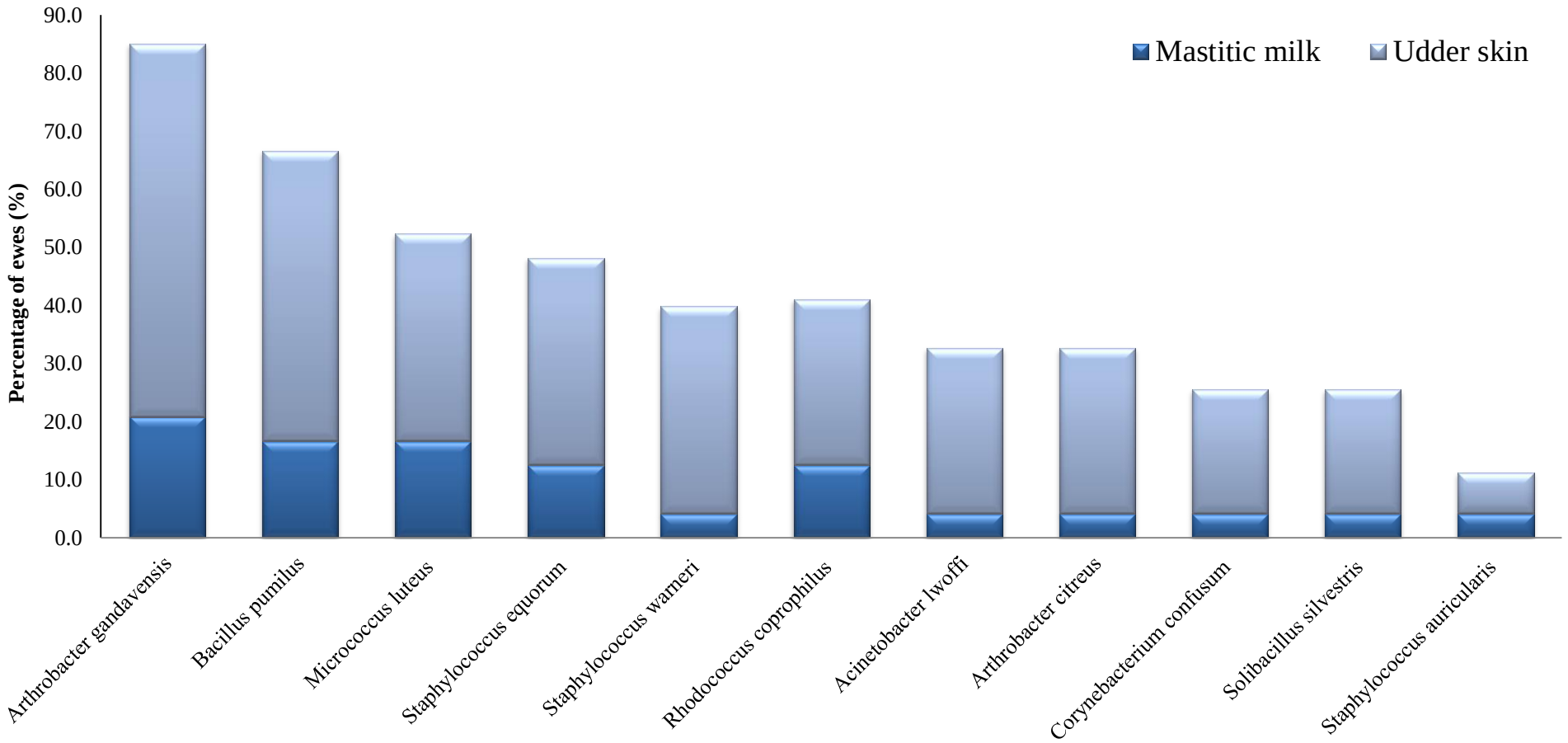


Figure 3.11: Percentage of ewes by species of bacteria present on udder skin and in milk from ewes with clinical mastitis.



3.4.2. Comparison of PFGE and MALDI-ToF-MS

The GelCompar dendrograms based on PFGE and the Biotyper 3.0 CCIs based on MALDI-ToF-MS spectra were aligned for *Staphylococcus warneri, Staphylococcus equorum, Bacillus pumilus, Micrococcus luteus* and *Rhodococcus corprophilus* (Figures 3.12-3.16). An example of the dendrograms produced in GelCompar alongside the PFGE gels and CCI matrices produced in Biotyper 3.0 is shown in Appendix 1 for the *Staphylococcus warneri* isolates.

For *Staphylococcus warneri* isolates shown in Figure 3.12, isolates 1-3 were 3 different strain types of *Staphylococcus warneri* and had very low CCI indices compared to all other *Staphylococcus warneri* isolates (mean=0.23, range=0.00-0.44). Alternatively, isolates 4-13 had the same pulsotype and had much higher CCI indices (mean=0.65, range=0.44-0.84). Isolate 4, although the same strain type was from a different ewe and from a different sample type- udder skin instead of milk.

For *Staphylococcus equorum* isolates (Figure 3.13), there was a wide range in strain types, with only 2 of the 14 isolates being the same strain type. MALDI-ToF-MS was able to differentiate between all isolates, and gave those most dissimilar according to PFGE the lowest CCI indices (isolates 1 and 2). The highest and only CCI index that corresponded to an identical pulsotype was 0.91. The remaining CCI indices ranged from 0.01-0.88 for isolates that were not the same strain type. This indicates that the spectra of different strain types are likely to be similar, and therefore the range and mean CCI of isolates with the same strain type are much higher than for other species, such as *Staphylococcus warneri* for example.

Only 2 strain types were isolated for *Rhodococccus corprophilus* isolates, 1-13 and 14-16, despite these isolates coming from 8 ewes from both udder skin and milk samples (Figure 3.14). Isolates 14-16 had CCI of 1.00 between each other- the highest possible CCI. The CCIs of these 3 isolates compared to the other strain type was low (mean=0.37, range=0.33-0.41). The majority of isolates were the other strain type, with CCI indices ranging from 0.55-0.97 with a mean of 0.75.

Like *Staphylococcus equorum, Bacillus pumilus* isolates were a range of strain types, apart from 2 isolates-7 and 8 which had the highest CCI of 0.77 (Figure 3.15). The remaining isolates had a range of CCIs from 0.13-0.65 with a mean of 0.34.

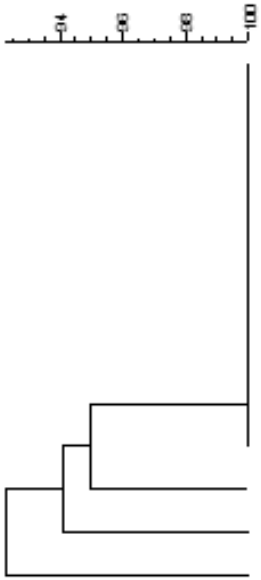
The majority of *Micrococcus luteus* isolates were one strain type and were isolated from the same ewes udder skin half (isolates 4-9) (Figure 3.16). The mean CCI index for these isolates were 0.88 (range 0.80-0.97). The 3 isolates that were unique strain types had a mean CCI of 0.59 (range 0.45-0.70).

A comparison of the mean and range of CCIs for isolates of the same and different strain types are shown in Table 3.6. For some isolates, such as *Rhodococcus corprophilus*, and *Micrococcus luteus* there appears to be an absolute cut-off- 0.41 and 0.70 respectively to determine strain types. For other isolates, such as *Staphyloccocus equorum* and *Bacillus pumilus*, there are distinct differences between the same and different strain types, but an absolute value is more difficult to obtain due to the low number of isolates that were the same strain type. *Staphylococcus equorum* has the largest range, and therefore the highest indices for isolates that are not the same strain type, followed by *Micrococcus luteus*. However, the CCIs for isolates with the same strain type are also highest indicating spectra of these species are more similar in general. In addition, there is no absolute cut-off between the same or different strain types for *Staphylococcus warneri* isolates, but rather a gradual increase in the likelihood of 2 isolates to be the same strain type as the CCI increases.

Table 3.6: Summaries of composite correlation indices for all species.

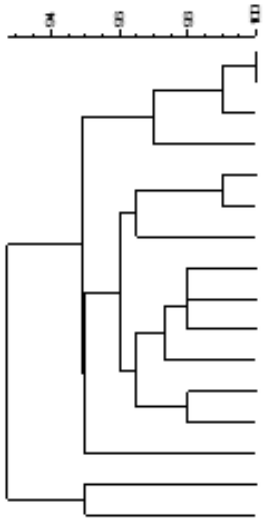
Bacterial species	Same strain type		Different strain type	
	Mean	Range	Mean	Range
Staphylococcus warneri	0.65	0.44-0.84	0.23	0.0-0.44
Staphylococcus equorum	0.91		0.40	0.01-0.88
Rhodococcus corprophilus	0.76	0.55-1.00	0.37	0.33-0.41
Bacillus pumilus	0.77		0.34	0.13-0.65
Micrococcus luteus	0.88	0.80-0.97	0.59	0.45-0.70

Figure 3.12: Comparison of Pulsed-Field Gel Electrophoresis derived dendrograms and colour coded Composite correlation indices for *Staphylococcus warneri* (green represents lowest estimates and red highest).



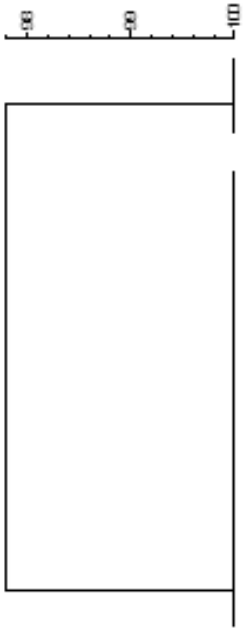
	1	2	3	4	5	6	7	8	9	10	11	12	13
13: 5048-1-2	0.18	0.40	0.39	0.50	0.66	0.82	0.68	0.82	0.84	0.83	0.69	0.67	1.00
12: 5048-1-1	0.00	0.35	0.05	0.68	0.49	0.60	0.78	0.60	0.62	0.61	0.76	1.00	0.67
11: 5048-1-3	0.01	0.32	0.07	0.65	0.52	0.63	0.80	0.63	0.66	0.64	1.00	0.76	0.69
10: 5048-1-4	0.18	0.38	0.33	0.53	0.71	0.82	0.59	0.76	0.78	1.00	0.64	0.61	0.83
9: 5048-1-5	0.18	0.38	0.35	0.44	0.60	0.79	0.67	0.79	1.00	0.78	0.66	0.62	0.84
8: 5048-1-6	0.15	0.36	0.37	0.43	0.61	0.79	0.65	1.00	0.79	0.76	0.63	0.60	0.82
7: 5048-1-7	0.00	0.29	0.05	0.55	0.43	0.64	1.00	0.65	0.67	0.59	0.80	0.78	0.68
6: 5048-1-8	0.19	0.38	0.39	0.48	0.64	1.00	0.64	0.79	0.79	0.82	0.63	0.60	0.82
5: 5048-1-9	0.16	0.44	0.32	0.43	1.00	0.64	0.43	0.61	0.60	0.71	0.52	0.49	0.66
4: 77R-11-1	0.00	0.32	0.00	1.00	0.43	0.48	0.55	0.43	0.44	0.53	0.65	0.68	0.50
3: 6011-7-1	0.00	0.28	1.00	0.00	0.32	0.39	0.05	0.37	0.35	0.33	0.07	0.05	0.39
2: 77R-48-1	0.33	1.00	0.28	0.32	0.44	0.38	0.29	0.36	0.38	0.38	0.32	0.35	0.40
1: 77L-42-1	1.00	0.33	0.00	0.00	0.16	0.19	0.00	0.15	0.18	0.18	0.01	0.00	0.18

Figure 3.13: Comparison of Pulsed-Field Gel Electrophoresis derived dendrograms and colour coded Composite correlation indices for *Staphylococcus equorum* (green represents lowest estimates and red highest).



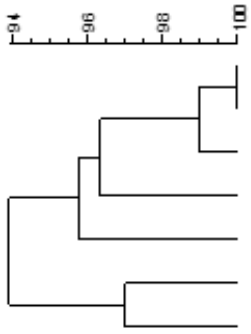
	1	2	3	4	5	6	7	8	9	10	11	12	13	14
14: 80-2-2	0.01	0.01	0.01	0.65	0.59	0.35	0.81	0.61	0.78	0.88	0.78	0.69	0.91	1.00
13: 80-2-1	0.01	0.01	0.03	0.61	0.65	0.36	0.77	0.59	0.79	0.78	0.81	0.70	1.00	0.91
12: 5070-13-3	0.01	0.01	0.01	0.41	0.69	0.25	0.67	0.46	0.61	0.58	0.72	1.00	0.70	0.69
11: 80-2-3	0.01	0.01	0.01	0.54	0.70	0.35	0.70	0.57	0.76	0.68	1.00	0.72	0.81	0.78
10: 59A-10-1	0.01	0.01	0.45	0.63	0.43	0.35	0.80	0.57	0.78	1.00	0.68	0.58	0.78	0.88
9: 5070-12-1	0.01	0.01	0.05	0.61	0.50	0.37	0.77	0.57	1.00	0.78	0.76	0.61	0.79	0.78
8: 59a-2-2	0.35	0.41	0.04	0.81	0.46	0.56	0.56	1.00	0.57	0.57	0.57	0.46	0.59	0.61
7: 80-2-7	0.01	0.01	0.14	0.66	0.49	0.31	1.00	0.56	0.77	0.80	0.70	0.67	0.77	0.81
6: 59a-3-1	0.25	0.42	0.47	0.44	0.27	1.00	0.31	0.56	0.37	0.35	0.35	0.25	0.36	0.35
5: 77R-9-1	0.01	0.01	0.01	0.39	1.00	0.27	0.49	0.46	0.50	0.43	0.70	0.69	0.65	0.59
4: 80-2-6	0.31	0.40	0.02	1.00	0.39	0.44	0.66	0.81	0.61	0.63	0.54	0.41	0.61	0.65
3: 77R-8-1	0.34	0.02	1.00	0.02	0.01	0.47	0.14	0.04	0.05	0.45	0.01	0.01	0.03	0.01
2: 59A-1-2	0.25	1.00	0.02	0.40	0.01	0.42	0.01	0.41	0.01	0.01	0.01	0.01	0.01	0.01
1: 77L-11-2	1.00	0.25	0.34	0.31	0.01	0.25	0.01	0.35	0.01	0.01	0.01	0.01	0.01	0.01

Figure 3.14: Comparison of Pulsed-Field Gel Electrophoresis derived dendrograms and colour coded Composite correlation indices for *Rhodococcus corprophilus* (green represents lowest estimates and red highest).



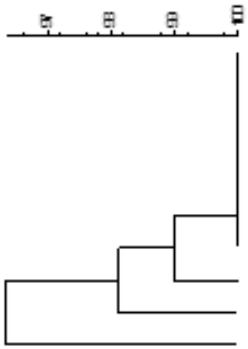
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
16: 54L-27-1	0.41	0.35	0.36	0.38	0.37	0.36	0.33	0.40	0.40	0.35	0.35	0.37	0.36	1.00	1.00	1.00
15: 5056-1-4	0.41	0.35	0.36	0.38	0.37	0.36	0.33	0.40	0.40	0.35	0.35	0.37	0.36	1.00	1.00	1.00
14: 5056-1-1	0.41	0.35	0.36	0.38	0.37	0.36	0.33	0.40	0.40	0.35	0.35	0.37	0.36	1.00	1.00	1.00
13: 77L-44-1	0.80	0.77	0.55	0.91	0.92	0.68	0.77	0.75	0.87	0.56	0.67	0.72	1.00	0.36	0.36	0.36
12: 62L-32-1	0.79	0.68	0.59	0.78	0.71	0.70	0.63	0.76	0.77	0.63	0.84	1.00	0.72	0.37	0.37	0.37
11: 62L-20-2	0.83	0.73	0.64	0.73	0.70	0.75	0.70	0.82	0.82	0.69	1.00	0.84	0.67	0.35	0.35	0.35
10: 59A-5-1	0.71	0.61	0.82	0.64	0.61	0.63	0.61	0.68	0.73	1.00	0.69	0.63	0.56	0.35	0.35	0.35
9: 54L-50-1	0.92	0.84	0.72	0.95	0.94	0.79	0.84	0.90	1.00	0.73	0.82	0.77	0.87	0.40	0.40	0.40
8: 54L-45-1	0.90	0.79	0.65	0.85	0.82	0.76	0.76	1.00	0.90	0.68	0.82	0.76	0.75	0.40	0.40	0.40
7: 5070-13-2	0.79	0.81	0.55	0.82	0.83	0.72	1.00	0.76	0.84	0.61	0.70	0.63	0.77	0.33	0.33	0.33
6: 5070-13-1	0.80	0.76	0.59	0.75	0.75	1.00	0.72	0.76	0.79	0.63	0.75	0.70	0.68	0.36	0.36	0.36
5: 5056-1-3	0.87	0.82	0.60	0.97	1.00	0.75	0.83	0.82	0.94	0.61	0.70	0.71	0.92	0.37	0.37	0.37
4: 5056-1-2	0.88	0.81	0.60	1.00	0.97	0.75	0.82	0.85	0.95	0.64	0.73	0.78	0.91	0.38	0.38	0.38
3: 4916-4-2	0.68	0.60	1.00	0.60	0.60	0.59	0.55	0.65	0.72	0.82	0.64	0.59	0.55	0.36	0.36	0.36
2: 4916-4-1	0.81	1.00	0.60	0.81	0.82	0.76	0.81	0.79	0.84	0.61	0.73	0.68	0.77	0.35	0.35	0.35
1: 27R-11-1	1.00	0.81	0.68	0.88	0.87	0.80	0.79	0.90	0.92	0.71	0.83	0.79	0.80	0.41	0.41	0.41

Figure 3.15: Comparison of Pulsed-Field Gel Electrophoresis derived dendrograms and colour coded Composite correlation indices for *Bacillus pumilus* (green represents lowest estimates and red highest).



7: 5070-8-3	0.30	0.13	0.61	0.35	0.44	0.77	1.00
6: 5070-8-2	0.34	0.13	0.65	0.32	0.45	1.00	0.77
5: 44-4-1	0.32	0.16	0.41	0.47	1.00	0.45	0.44
4: 4916-1-1	0.35	0.16	0.42	1.00	0.47	0.32	0.35
3: 6011-9-1	0.31	0.16	1.00	0.42	0.41	0.65	0.61
2: 62L-35-1	0.31	1.00	0.16	0.16	0.16	0.13	0.13
1: 62L-26-1	1.00	0.31	0.31	0.35	0.32	0.34	0.30
	1	**2**	**3**	**4**	**5**	**6**	**7**

Figure 3.16: Comparison of Pulsed-Field Gel Electrophoresis derived dendrograms and colour coded Composite correlation indices for *Micrococcus luteus* (green represents lowest estimates and red highest).



9: 27L-2-8	0.52	0.66	0.51	0.80	0.88	0.89	0.88	0.92	1.00
8: 27L-2-7	0.57	0.73	0.54	0.86	0.97	0.90	0.95	1.00	0.92
7: 27L-2-6	0.58	0.72	0.55	0.83	0.93	0.91	1.00	0.95	0.88
6: 27L-2-5	0.52	0.68	0.50	0.80	0.86	1.00	0.91	0.90	0.89
5: 27L-2-3	0.56	0.70	0.53	0.85	1.00	0.86	0.93	0.97	0.88
4: 27L-1-8	0.68	0.64	0.48	1.00	0.85	0.80	0.83	0.86	0.80
3: 4956-3-1	0.53	0.64	1.00	0.48	0.53	0.50	0.55	0.54	0.51
2: 30-2-1	0.45	1.00	0.64	0.64	0.70	0.68	0.72	0.73	0.66
1: 27L-1-1	1.00	0.45	0.53	0.68	0.56	0.52	0.58	0.57	0.52
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**

Bacillus pumilus (6 strains) and *Staphylococcus equorum* (15 strains) were the most varied in terms of strain types while only 2, 4 and 4 strain types were isolated from all ewes for *Rhodococcus corprophilus*, *Staphylococcus warneri* and *Micrococcus luteus* respectively. Only for *Staphylococcus warneri* (Figure 3.12, isolates 5-13) and *Micrococcus luteus* (Figure 3.16 isolates 4-9) were the majority of morphologically identical isolates found to be the same strain type. For all other species, despite picking sets of isolates that were morphologically similar these represented several different strains (Figure 3.13, isolates 4, 7, 11, 13 and 14 and Figure 3.14 isolates 4, 5, 14 and 15). This indicates the necessity to pick multiple isolates with the same morphology in order to isolate a variety of strain types during epidemiological studies.

Interestingly, milk samples from ewes without clinical signs of mastitis had up to 3 species present per sample. This is very different to samples from ewes with clinical mastitis, where a variety of species were isolated from generally more diverse cultures.

The same strain type was found in mastitic milk and on udder skin for *Staphylococcus warneri* (Figure 3.12, isolates 5-13 from milk and isolate 4 from skin) and *Rhodococcus corprophilus* (Figure 3.14, isolates 1, 8-13 from milk and isolates 2-7 from skin; isolate 16 from milk and isolates 14-15 from skin).

3.5. Discussion

This study aimed to use MALDI-ToF-MS to investigate reservoirs of mastitis causing pathogens in suckler ewes. At the start of this study, it was unknown how successful MALDI-ToF-MS would be in the identification of mastitis causing pathogens to species level and isolates from the environment of the ewe. During the course of this study, MALDI-ToF-MS started to be used in bacterial identification of mastitis pathogens in cows (Barreiro *et al.* 2010, El Behiry *et al.* 2014) and so far has proved to be mostly successful for clinical veterinary isolates. However, the MALDI-ToF-MS database is populated with isolates from human clinical samples, and as the identification method is based on pattern matching this bias meant only 67% (504/752 isolates, Table 3.5) of isolates were successfully identified. Despite this, only a small proportion of isolates (0.6%: Table 3.5) were not assigned spectra.

The remaining unidentified or poorly identified isolates (~32%) may become identifiable as the database improves in the future. These results indicate that, with an improved spectral database, up to 98% of isolates from ewes could potentially be identified to at least species level. However, this is a current limitation of MALDI-ToF-MS in that isolates unidentified could be important novel pathogens.

This study also aimed to assess the use of MALDI-ToF-MS as a method of strain differentiation by comparing it to the current gold standard strain typing method for staphylococci, PFGE. For strain clustering, the database is less vital as strains are compared to one another rather than the database. In order to do this, the methods used to apply PFGE to some of these species also needed to be created. For example, no studies on PFGE of the *Rhodococcus* genus existed. For the other species in this study, some papers were available for PFGE protocols for *Micrococcus* (Murayama *et al.* 2003), *Bacillus* (Zhong *et al.* 2007) and *Staphylococcus* species, in particular, *Staphylococcus aureus* (He *et al.* 2014). In this study, an established PFGE method for *Staphylococcus aureus* (McDougal *et al.* 2003) was successfully optimised to be used for all species observed in this study.

MALDI-ToF-MS had good correspondence with PFGE for all species observed in this study including *Staphylococcus warneri, Staphylococcus equorum, Rhodococcus corprophilus, Bacillus pumilus* and Micrococcus *luteus*. This supports the current literature where MALDI-ToF-MS has been compared to a variety of strain typing methods including multi locus sequence typing (MLST) (Nagy *et al.* 2013), REP-PCR (Siegrist *et al.* 2007) and 16S rRNA gene sequencing (Rettinger *et al.* 2012). MALDI-ToF-MS has also shown good correspondence to PFGE for *Vibrio* (Eddabra *et al.* 2012), and *Legionella* (Fujinami *et al.* 2011) strains.

Unlike many of the previous studies on the use of MALDI-ToF-MS for strain differentiation, which used dendrograms produced by the associated software, this study utilised CCI values to compare MALDI-ToF-MS to PFGE. This was deemed more accurate as the CCI values are absolute between each isolate pair whereas the dendrograms produced in Biotyper 3.0 can alter drastically depending on the strain types inputted into the software as they are built based on pairs of similar isolates.

The mean CCI for isolates of the same and different strain types varied between species which is to be expected as the spectra of some species vary more than others. In general, however, the mean CCI for isolates of different strain types ranged from 0.23-0.59 whereas the mean CCI for isolates of the same strain type ranged from 0.65-0.91. To the author's knowledge, no other study has compared MALDI-ToF-MS CCI values to another strain typing method, and therefore these values cannot be compared to the literature. However, the correspondence of these values to PFGE suggests it may be possible to produce cut-off estimates for use with MALDI-ToF-MS for each bacterial species to determine whether two isolates are the same strain type. The CCI values in this study show a clear difference between isolates that are the same and different strain types except for *Staphylococcus warneri* where the threshold for isolates of the same and different strain types meet at 0.44. This difference was seen even though the 'direct method' was used which can produce more background noise in the mass spectral fingerprints. Future work would benefit from using a formic extraction method which would reduce noise, increase peak intensity and therefore result in reduced CCI variability.

Over 130 species have been associated with bovine mastitis (Watts 1988). It therefore seems likely that a similar number of organisms will be associated with ovine mastitis. Several species are often focussed on in mastitis epidemiological research, with other frequently isolated species being ignored as contamination. If these species are often present on udder skin, in lamb mouths and in ewe milk, it could indicate these species are associated with mastitis, and provide evidence for their reservoirs.

A smaller number of species isolated per milk sample tended to be observed in ewes without any clinical signs of mastitis. It was of particular interest that *Escherichia coli, Streptococcus uberis* and *Mannhaemia haemolytica* were present in samples from ewes without clinical signs samples, but were not present in the samples taken from ewes with clinical signs.

A wide range of species were isolated from milk samples from ewes with clinical mastitis, the most frequently isolated including *Arthrobacter gandavensis, Bacillus pumilus*, and *Micrococcus luteus.* To the author's knowledge, there are no published data on the presence of *Arthrobacter gandavensis* and *Micrococcus luteus* in sheep

milk on or udder skin. Although the importance of toxigenic *Bacillus pumilus* has been highlighted in cow milk (Nieminen *et al.* 2007).

Given that the majority of mastitis causing pathogens are thought to enter the mammary gland through the teat canal, bacteria present on teat apices, udder skin and in teat lesions are likely to play an important role in the development of mastitis. If these bacteria are able to persist on the udder skin and enter the mammary gland, causing infection, the same strain types will be found in both environments. The same strain type was found in mastitic milk and on udder skin for *Staphylococcus warneri* and *Rhodococcus corprophilus* indicating a possible reservoir of bacteria that might enter the mammary gland. *Staphylococcus warneri* has been found to cause intramammary infections in cows previously (Gillespie *et al.* 2009, Mørk *et al.* 2012a, Rajala-Schultz *et al.* 2009, Taponen *et al.* 2006, Waller *et al.* 2011).

There are few studies on reservoirs of mastitis-causing pathogens in sheep, with these mostly being focussed on *Staphylococcus aureus* (Mørk *et al.* 2012b). A study of repeated sampling of milk, teat skin, nasal and vaginal mucous membrane swabs for 3 suckler flocks found *Staphylococcus aureus* was frequently isolated from the mucous membranes of the nose of suckler sheep and lambs. Furthermore, the majority of isolates from the nasal cavity of mothers, lambs and twins were identical or different by ≤3 bands using PFGE (Mørk *et al.* 2012b, Vautor *et al.* 2005a). There were, however several limitations to this study, namely that there was a dominating PFGE pulsotype in the flock and furthermore the definition of a pulsotype being different was by ≤3 bands. This could have resulted in isolates of different strain types being defined as the same, inaccurately identifying reservoirs. In this study, a change in 1 band was used to define a new strain type making the analysis here more conservative. There is still some debate over the number of band differences that define a difference in strain type (Tenover *et al.* 1995).

Some bacterial species varied in terms of strain types. *Bacillus pumilus* and *Staphylococcus equorum* were the most varied in terms of strain types. Traditionally the literature has considered *Staphylococcus equorum* as an 'environmental' organism (Kloos & Schleifer 1986, Piessens *et al.* 2012). This diversity of strains suggests there are many sources of *Staphylococcus equorum* which fits the traditional view of this organism having an "environmental" origin from many

different sources. Conversely, only 2-4 strain types were isolated from all 27 ewes for *Rhodococcus corprophilus*, *Staphylococcus warneri* and *Micrococcus luteus* respectively indicating fewer sources of these species and suggests either there are few predominant strains in the environment or that they are more "contagious" in nature and pass from ewe to ewe.

In this study, up to 9 morphologically identical isolates were isolated from each milk sample, and up to 5 from the more diverse communities from udder skin samples. However, in a longitudinal study where larger numbers of ewes and sampling points are required, this may not be possible. In addition, due to the full coverage of growth on agar plates, and therefore difficulties in distinguishing individual colonies on plates, particularly for udder skin samples, sterile streaking may be necessary for future studies on reservoirs and transmission pathways of mastitis causing bacteria. In addition selective media could be used in order to target the isolation of specific species associated with mastitis to test transmission events more successfully. However MALDI-ToF-MS would need to be optimised in order to deal with alternative agar and the use of a formic acid extraction method for sample preparation for MALDI-ToF-MS would improve identifications and minimise background peaks and noise.

3.6. Conclusion

This study successfully utilised MALDI-ToF-MS to identify the majority of bacteria isolated from milk and udder skin samples to the species level. MALDI-ToF-MS had good correspondence at the strain level with PFGE for all species observed in this study including *Staphylococcus warneri, Staphylococcus equorum, Rhodococcus corprophilus, Bacillus pumilus* and *Micrococcus luteus*. The same strain type was found in mastitic milk and on udder skin for *Staphylococcus warneri* and *Rhodococcus corprophilus* indicating that udder skin might act as a reservoir for mastitis causing pathogens.

Chapter 4. The identification of potential transmission pathways of intramammary infections using MALDI-ToF-MS

4.1. Introduction

As highlighted in Chapter 3, strain typing is necessary to identify reservoirs of mastitis causing bacteria. However, in order to ascertain whether a transmission event has occurred, the same strain must be isolated from multiple environments over time.

Previous research on the transmission and persistence of mastitis causing bacteria from different environments of ewes is lacking. In dairy cows, research on reservoirs and/or routes of transmission have mostly focussed on *Staphylococcus aureus.*

In dairy cows, potential reservoirs of *Staphylococcus aureus* have been identified; heifers with teat skin colonised by *Staphylococcus aureus* were more likely to have intramammary infections caused by *Staphylococcus aureus* at parturition (Roberson *et al.* 1994). In addition, using pulsed-field gel electrophoresis (PFGE), the same pulsotypes of *Staphylococcus aureus* were identified from teat skin as in milk in several studies (da Costa *et al.* 2014, Haveri *et al.* 2008, Zadoks *et al.* 2002). PFGE has also been used to identify the same *Staphylococcus aureus* pulsotypes from body sites (particularly the hock skin), the immediate environment of lactating cows and milk samples (Capurro *et al.* 2010). Strains of *Staphylococcus aureus* predominant on some farms were also isolated from flies collected in the barns of lactating cows and milking equipment (Capurro *et al.* 2010). A study with extramammary sites including dairy cow teat skin, teat canals, skin lesions, milking liners and the hands and nostrils of milking personnel within two dairy herds found indistinguishable *Staphylococcus aureus* pulsotypes for isolates infecting the mammary gland and extramammary sites, again indicating potential reservoirs of intramammary infections (Haveri *et al.* 2008). However, the direction of transmission could not be ascertained in this case.

In sheep, identical *Staphylococcus aureus* pulsotypes were identified from body sites and in the milk. In particular the majority of pairs of isolates from the nasal cavity of

ewes and their lambs, twins and repeated sampling provided identical pulsotypes indicating transmission of *Staphylococcus aureus* between the dam and her lambs (Mørk *et al.* 2012b). However, this could also be due to the predominance of a particular strain type within these flocks, and therefore does not necessarily indicate a transmission event has occurred. *Mannheimia haemolytica* was present on ewe udder skin post-lambing but not pre-lambing, and was isolated from lamb mouths indicating that *Mannheimia haemolytica* may be transmitted from the mouth of the lambs onto the teat of the dam during sucking (Scott & Jones 1998). However, strain typing methods were not used in this study and therefore transmission could not be confirmed.

Alongside transmission events, there is also the possibility for the same isolates to persist in an environment over time. For example, there is some evidence for persistent infections in the bovine udder caused by *Escherichia coli* (Bradley & Green 2001), Enterobacterial isolates through the dry period (Bradley & Green 2000), and *Streptococcus uberis* (Abureema *et al.* 2014). These studies utilised strain typing methods in order to ascertain whether the same strain types were present in an environment over time. However to the author's knowledge there are no studies on the persistence of the same strain types in the milk or on the udder skin of ewes.

In suckler ewes, there is a lack of knowledge on transmission pathways and the persistence of mastitis causing bacteria.

4.2. Aims

The aim of this study was to identify potential transmission events and persistence of the same bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time. This aim was addressed by optimising MALDI-ToF-MS sample preparation and analysis in BioTyper 3.0 and FlexAnalysis to be utilised for strain differentiation and overcoming the spectral variability associated with media type.

4.3. Methods

4.3.1. Flock and animals

A farm in Shropshire, England was selected as the study farm. Forty-six ewes comprising 20 3 year old ewes and 23 6 year old and 3 2 year old ewes were randomly selected for milk sampling at time of enrolment and the following time points: 1-4 days of lambing, at approximately 4 weeks and 8 weeks into lactation.

4.3.2. Sampling

Milk samples for bacteriology were aseptically collected by Selene Huntley according to the methodology described in Chapter 3. Swab samples were taken according to the methodology described in Chapter 3. Both milk and udder skin swabs were taken from all ewes at 1-4 days after lambing, and approximately 4 weeks and 8 weeks into lactation.

For lamb mouth samples, swabs were inserted to the back of the lambs' throat and twisted on each side at the first observation only (1-4 days of lambing).

Milk (with glycerol added to 20% v/v), udder skin and lamb mouth swab samples were stored and transported on ice to the laboratory (University of Warwick). Swabs were immediately broken off into 1ml of Brain Heart Infusion agar (BHI) and glycerol (at 10% v/v) into a cryovial tube. They were then stored at -20°C until analysis.

4.3.3. Growth and selection of isolates from agar plates

A random selection of these samples were taken for laboratory analysis across all three age groups: 8 3 year old ewes and 8 6 year old and 3 2 year old ewes.

Each sample was thawed at room temperature and plated onto three different types of agar using aseptic techniques; a sterile 10µl loop was used to sterile streak onto half a BHI + sheep's blood agar (SBA) plate containing 5% (v/v) sterile sheep's blood and a new sterile 10µl loop for half an Edwards agar plate (a selective medium for isolation of streptocococi typically involved in bovine mastitis) (Oxoid 2014a). In addition, 100µl was pipetted onto a MacConkey agar plate (a selective medium

for isolation of Gram-negative and enteric bacilli) (Oxoid 2014b) and spread. The plates were incubated inverted at 37°C. Plates were checked at 24, 48 and 72 hours, and colonies picked as described below.

Milk

Due to the low numbers of isolates cultured from milk samples, the majority of isolates were picked under the supervision of an experienced microbiologist, and in particular those typically morphologically associated with mastitis. Morphologically identical isolates were not found as frequently as in Chapter 3, apart from where there was pure growth of one species. However, in all cases, up to 5 of each morphologically unique isolate was picked for further analysis.

Udder skin swabs

The altered plating technique (using three plates, two of which are selective media and sterile streaking onto SBA plates) provided a more efficient method for picking off isolates of interest. Under the supervision of an experienced microbiologist, isolates that were morphologically similar to isolates picked from their milk and/or lamb mouth counterparts were selected for further analysis.

Lamb mouth swabs

As growth was limited to a single morphologically distinct colony type from most lamb mouth swab samples, up to 5 of each morphologically unique isolate were picked for further analysis regardless of whether these were also identified in the milk or on udder skin.

Isolate storage

Once selected, isolates were quarter streaked as in Chapter 3.However, plates were labelled with sample number (a unique 5 digit number for each udder half), the sample type (S-Skin, Blank-Milk, L-Lamb), the media in which they were isolated from (MC-MacConkey, ED-Edwards, BD- SBA) and a unique letter (A-P). For example a *Staphylococcus aureus* isolate from an SBA plate might be labelled 43472 BD A. As there was no milk sample number pre-lambing, samples were labelled

with the ewe number, lamb number (if the sample came from a lamb mouth), the media in which they were isolated from as above, and a unique letter as above.

In order to reduce spectral variability, they were only passaged once before MALDI-ToF-MS analysis. This quadrant was also used to take culture for long-term storage in BHI and glycerol (at 10% v/v) at -80°C as in Chapter 3.

4.3.4. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

Optimisation of MALDI-ToF-MS sample preparation

Substantial efforts went into assessing and minimising spectral variability between technical replicates and media types to ensure strain differentiation would be accurate. Technical replicate variability was tested using *Staphylococcus aureus, Streptococcus dysglactiae* and *Streptococcus uberis*.

The media types tested were SBA (the standard agar used for MALDI-ToF-MS), MacConkey and Edwards agar, using coagulase-negative staphylococci for SBA and MacConkey agar and *Streptococcus uberis* for Edwards agar as these were the media types that would be used in this study.

MALDI-ToF-MS protocol

The reagent and sample set up was as described in Chapter 3. The bacterial test standard (BTS) (Bruker) was used as a control. Extracted isolates were spotted in duplicate on a 96 well Bruker target plate and run through MALDI-ToF-MS. Spectra were compared to the Biotyper 3.0 database and a species name and score were assigned (as in Chapter 3).

Spectral analysis

Species found in abundance on udder skin, in lambs mouths and ewe milk were taken forward for further software analysis (and therefore strain differentiation) using Biotyper 3.0, FlexAnalysis and ClinProTools. The results were saved in html format and transferred into Microsoft Access.

4.3.5. Data management

Two databases were constructed; one containing ewe data such as observation date, ewe ID, body condition score, lambing date, lamb ID, sex, and litter size. The second database contained the results from MALDI-ToF-MS analysis in order to select isolates of interest for potential transmission events.

These included isolates where the same species were identified in:

1) The same location for the same ewe over 2 or more consecutive observations (Potential bacterial persistence)

2) Two different environments for the same ewe (ewe milk, udder skin and/or the ewe's lamb's mouth) at the same time point (Potential reservoir or transmission pathway of unknown direction)

3) Two different environments for the same ewe (ewe milk, udder skin and/or the ewe's lamb's mouth) over 2 or more consecutive observations (Potential transmission pathway)

4) The same environments for different ewes (Potential transmission pathway)

Queries were used to merge technical replicates (Microsoft Access), allowing the top score for each isolate to be selected. Score value calculations are defined in Chapter 3. Queries were used to identify ewes where the same species were present on the udder skin and/or lamb mouths and at the next observation in the milk, or vise versa or where species persisted in the milk or on the skin over time.

Composite correlation indices (CCI) were calculated for spectra of interest using Biotyper 3.0 (described in Chapter 3) in order to select isolates that were likely to be the same strain types within samples from the same ewe and spectra from these isolates were manually compared using FlexAnalysis and ClinProTools. CCI matrices were exported to Micrososft Excel and conditional formatting was applied to produce a heat map for easy viewing.

4.4. Results

4.4.1. MALDI-ToF-MS optimisation

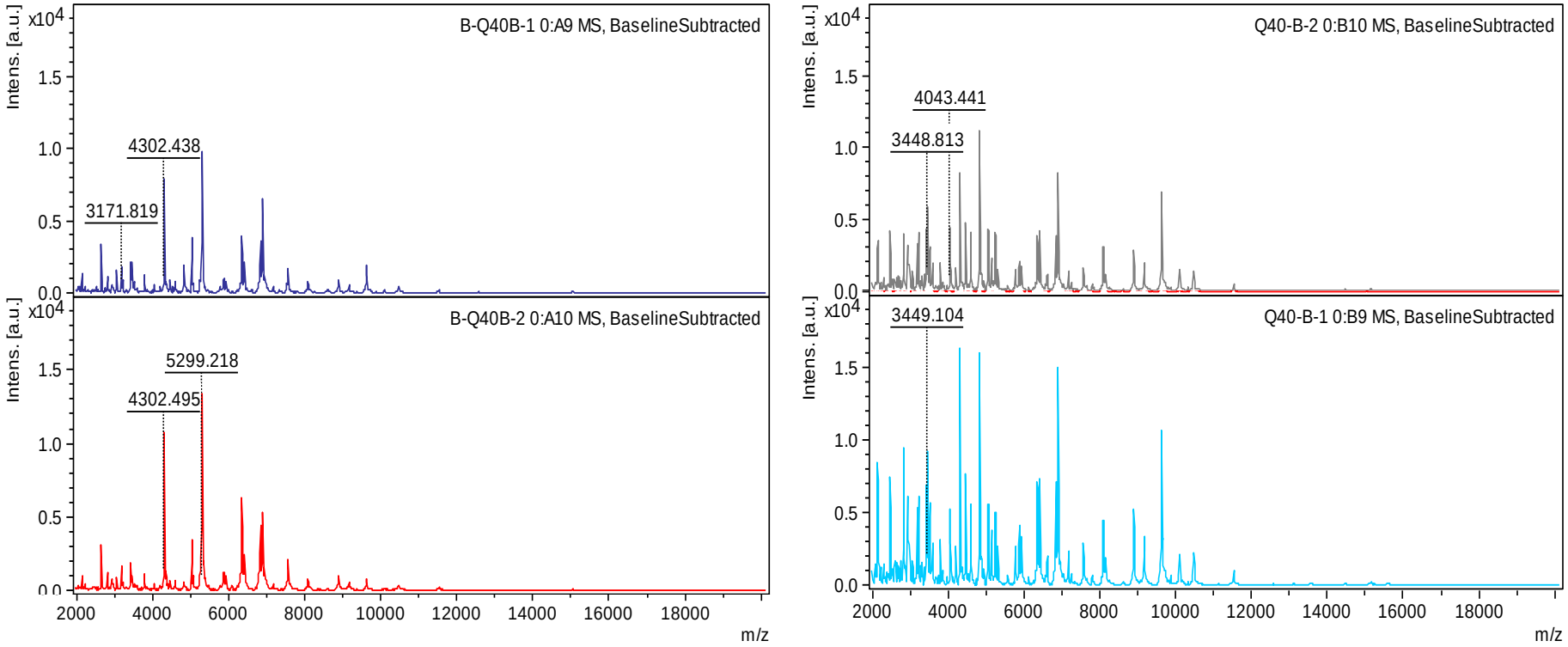
Although in Chapter 3 MALDI-ToF-MS was used for strain differentiation, the CCI thresholds for strain types could be more clearly defined using an extraction method by improving peak intensity and clarity and reducing background peaks.

For example, using a *Staphylococcus aureus* isolate with a direct and extracted method showed a difference in the number of peaks, average intensity of these peaks, quality factor and resolution (Table 4.1, Figure 4.1).

Table 4.1: Average intensity, quality factor and resolution of peaks from an extracted and direct method of sample preparation for a *Staphylococcus aureus* isolate.

	Average			
	No. peaks	Intensity	Quality Factor	Resolution
Direct	3	763.02	1351.95	819.67
Extracted	7	1708.67	2891.31	1001.85

Figure 4.1: Examples of spectra from duplicate samples of a *Staphylococcus aureus* isolate that were prepared using a direct smear (left) and extraction (right).



The extraction technique resulted in very high CCI estimates for 6 technical replicates for 3 test isolates grown on SBA: 0.95 -1.00 for *Staphylococcus aureus* and 0.96-1.00 for *Streptococcus agalactiae* and 0.96-1.00 *Streptococcus uberis* (Table 4.2, 4.3 and 4.4 respectively). An example of the spectra of technical replicates for *Streptococcus uberis* (CCI estimates shown in Table 4.4) are shown in Appendix 2.

Table 4.2: Composite correlation indices matrix for 6 technical replicates of *Staphylococcus aureus.*

Replicate 6						1.00
Replicate 5					1.00	0.97
Replicate 4				1.00	0.99	0.96
Replicate 3			1.00	0.96	0.95	1.00
Replicate 2		1.00	0.99	0.95	0.97	1.00
Replicate 1	1.00	0.96	0.96	1.00	1.00	0.97
	1	2	3	4	5	6

Table 4.3: Composite correlation indices matrix for 6 technical replicates of *Streptococcus agalactiae.*

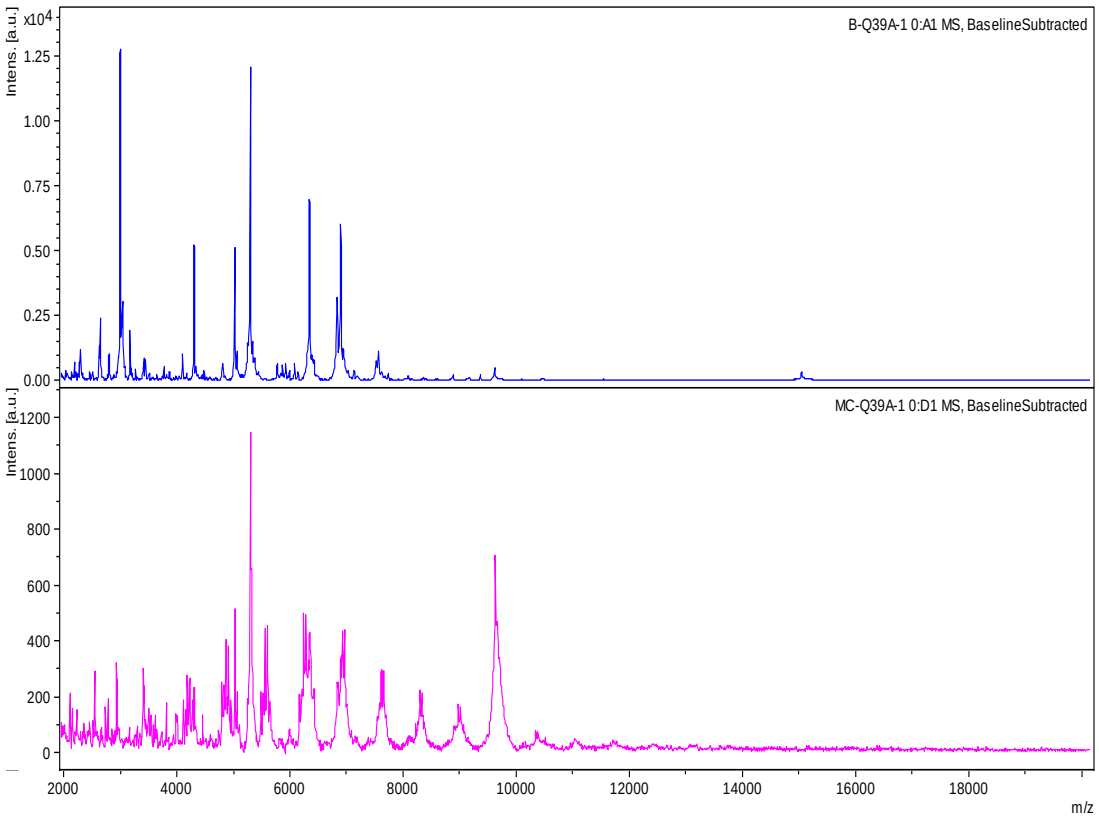
Replicate 6						1.00
Replicate 5					1.00	1.00
Replicate 4				1.00	1.00	1.00
Replicate 3			1.00	1.00	1.00	1.00
Replicate 2		1.00	1.00	1.00	1.00	1.00
Replicate 1	1.00	0.98	0.96	0.96	0.96	0.97
	1	2	3	4	5	6

Table 4.4: Composite correlation indices matrix for 6 technical replicates of *Streptococcus uberis.*

Replicate 6						1.00
Replicate 5					1.00	1.00
Replicate 4				1.00	1.00	1.00
Replicate 3			1.00	0.96	0.96	0.96
Replicate 2		1.00	0.96	1.00	1.00	1.00
Replicate 1	1.00	1.00	0.95	1.00	1.00	1.00
	1	2	3	4	5	6

There was a difference in peak presence and intensity for spectra obtained from the same isolate grown on two different media types (Figure 4.2). For example where *Staphylococcus aureus* was grown on SBA and MacConkey agar, not only was the intensity of all peaks drastically reduced, the spectral fingerprints were also different (Figure 4.2). This was also the case for Edwards agar.

Figure 4.2: Spectra from the same *Staphylococcus aureus* isolate from SBA (blue) and MacConkey agar (pink).



This would have been problematic as the study required different media types to be used. This problem was overcome by the addition of formic acid in the extraction protocol which improved peak intensity and adjusted peak shifts between media. This is shown in Figure 4.3 for MacConkey (coagulase-negative staphylococci), Figure 4.4 for SBA, (coagulase-negative staphylococci) and Figure 4.5 for Edwards agar (*Streptococcus uberis*) treated with and without a 70% formic acid overlay.

Figure 4.3: Differences in spectra from isolates treated with (green) and without (blue) an overlay of formic acid on MacConkey (coagulase-negative staphylococci).

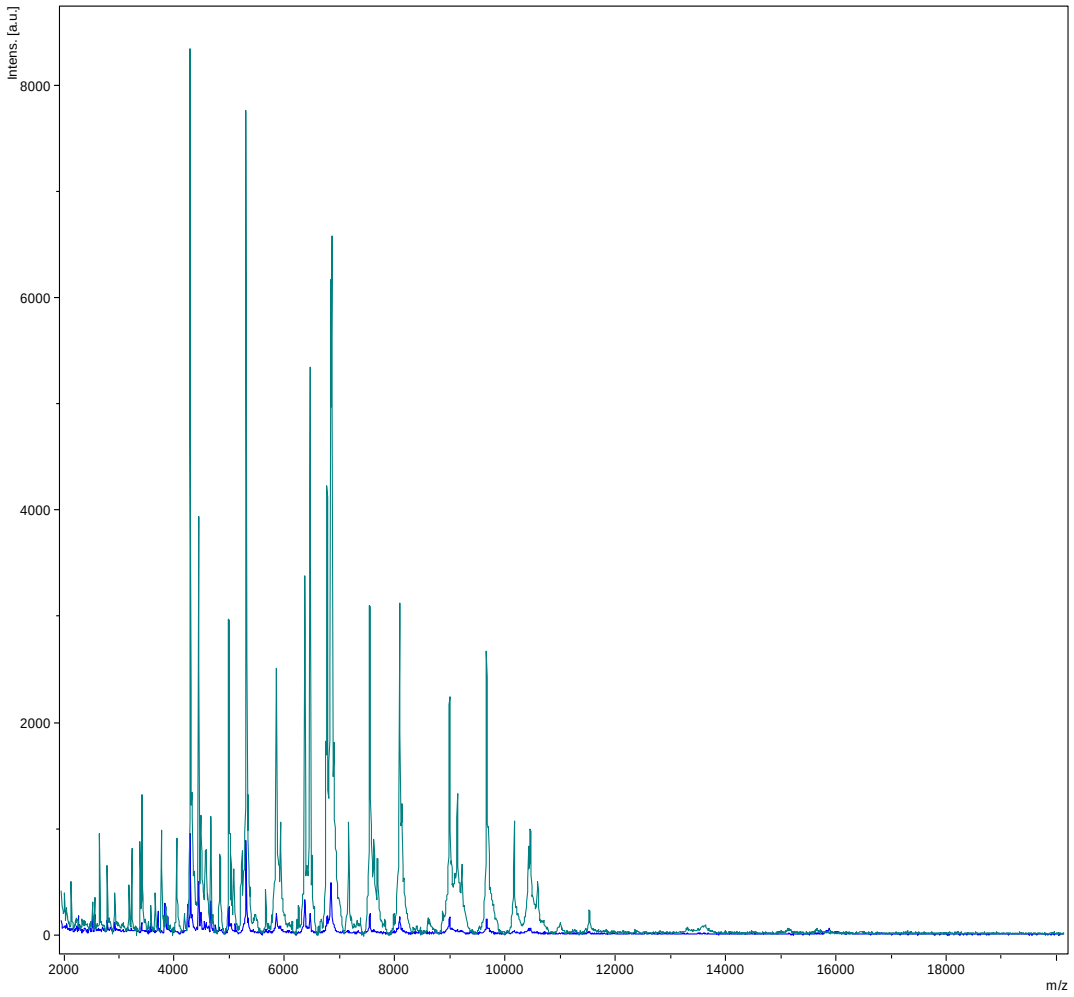


Figure 4.4: Differences in spectra from isolates treated with (green) and without (pink) an overlay of formic acid on SBA (coagulase-negative staphylococci).

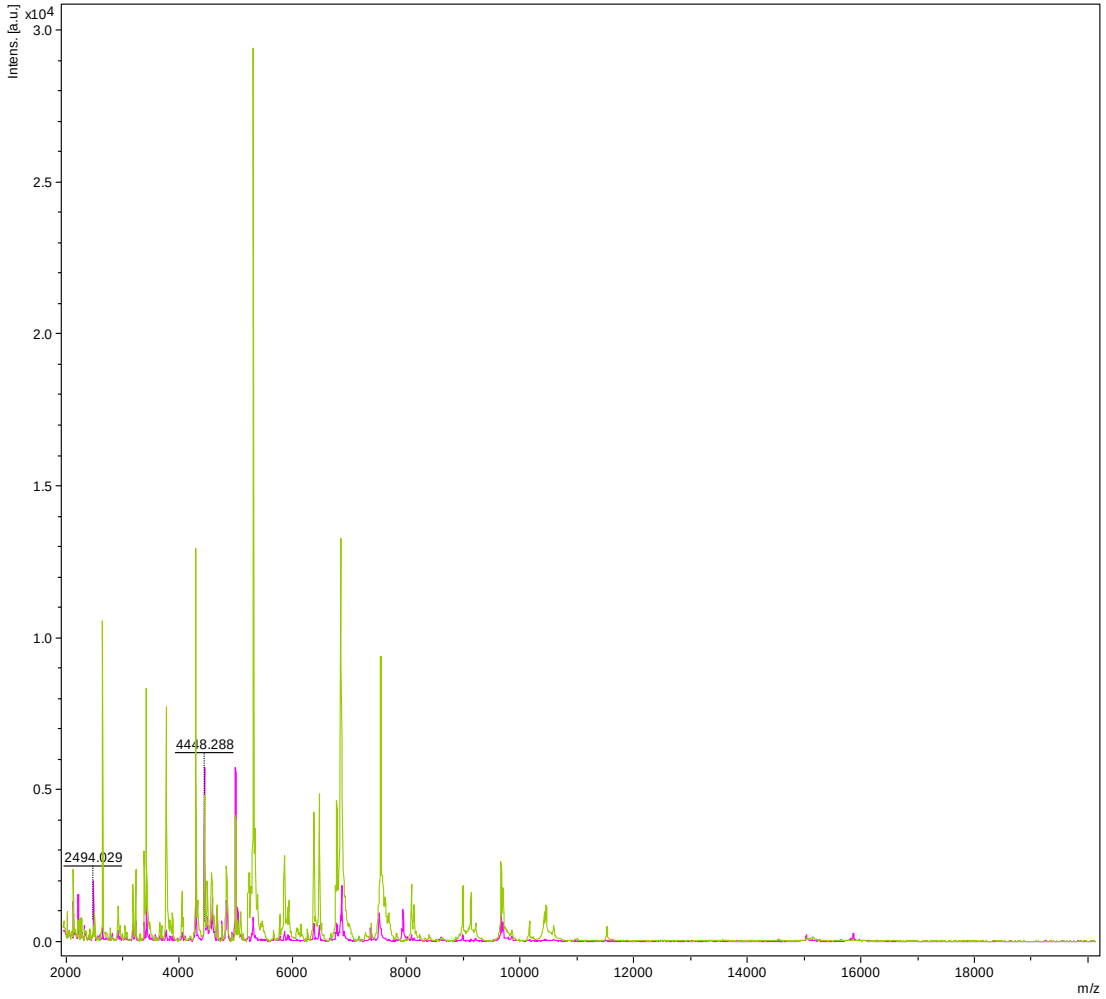
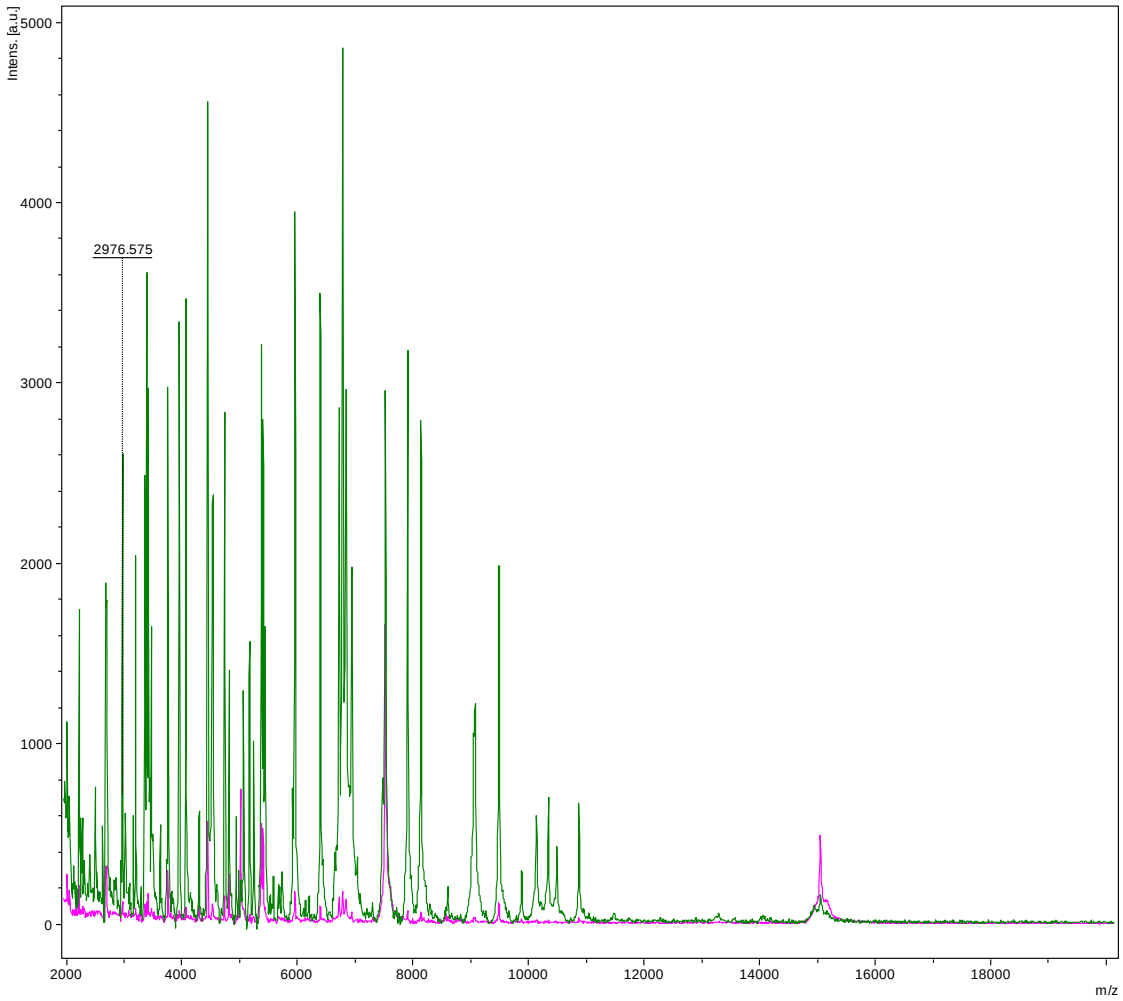


Figure 4.5: Differences in spectra from isolates treated with (green) and without (pink) an overlay of formic acid on Edwards agar (*Streptococcus uberis*).



4.4.2. Locations of isolated bacterial species

There were 287 milk and swab samples in total, from which 912 isolates were picked. A total of 1888 spectra were produced from these 912 isolates. Of the 912 isolates, 662 were assigned a suitable ID (score of ≥1.7) by MALDI-ToF-MS. The remaining isolates were not included in further analysis.

The species isolated from ewe milk and lamb mouths and the percentage of samples in which they were isolated are shown in Tables 4.5 and 4.6.

A number of species associated with mastitis were found in lamb mouths. For example, *Aerococcus viridans, Bacillus licheniformis, Escherichia coli, Enterococcus faecalis, Enterococcus faecium* and coagulase-negative staphylococci were isolated from lamb mouths (Table 4.5).

Several bacterial species associated with mastitis were isolated from ewe milk samples, including: *Acinetobacter baumannii, Aerococcus viridans, Bacillus cereus, Bacillus licheniformis, Kocuria kristinae, Staphylococcus aureus* and many coagulase-negative staphylococci (Table 4.6).

Twenty-six species were found in more than one location (lamb mouth, ewe udder skin and/or ewe milk) (Figure 4.6). *Staphylococcus equorum* was the most frequently isolated species from lamb mouths and udder skin and was also isolated from ewe milk. *Bacillus licheniformis, Aerococcus viridans, Staphylococcus warneri* and *Staphylococcus simulans* were also found in all three locations.

Escherichia coli, Staphylococcus vitulinus and Staphylococcus succinus were only found in lamb mouths and on ewe udder skin.

Micrococcus luteus, Staphylococcus epidermidis, Staphylococcus hominis, Enterococcus faecium and *Staphylococcus auricularis* were the most commonly isolated species from only udder skin and milk.

Although the summary of skin samples shown in Figure 4.6 includes species isolated from the udder skin pre and post lambing, there were some differences between these two time points (Table 4.7). For example, species typically associated with mastitis including: *Aerococcus viridans, Bacillus cereus, Enterococcus casseliflavus,*

Escherichia coli, Staphylococcus aureus, Staphylococcus chromogenes, Staphylococcus haemolyticus and *Streptococcus uberis* were all isolated from udder skin samples after lambing but not before.

Table 4.5: The percentage of milk samples containing each bacterial species (*n*=102).

Bacterial Species	Milk
Micrococcus luteus	100
Staphylococcus epidermidis	47.37
Bacillus licheniformis	42.11
Staphylococcus hominis	31.58
Staphylococcus equorum	15.79
Acinetobacter baumannii	10.53
Enterococcus faecium	10.53
Pseudomonas stutzeri	10.53
Staphylococcus haemolyticus	10.53
Staphylococcus saprophyticus	10.53
Staphylococcus warneri	10.53
Streptococcus salivarius	10.53
Abiotrophia defectiva	5.26
Acinetobacter lwoffii	5.26
Aerococcus viridans	5.26
Arthrobacter gandavensis	5.26
Bacillus cereus	5.26
Bacillus circulans	5.26
Bacillus sonorensis	5.26
Brevibacterium casei	5.26
Granulicatella elegans	5.26
Kocuria kristinae	5.26
Kocuria rhizophila	5.26
Lactobacillus paracasei	5.26
Lactobacillus sakei	5.26
Neisseria macacae	5.26
Neisseria mucosa	5.26
Neisseria perflava	5.26
Paenibacillus barengoltzii	5.26
Rothia mucilaginosa	5.26
Sphingomonas paucimobilis	5.26
Staphylococcus aureus	5.26
Staphylococcus auricularis	5.26
Staphylococcus caprae	5.26
Staphylococcus simulans	5.26
Streptococcus oralis	5.26
Streptococcus parasanguinis	5.26
Streptococcus sanguinis	5.26
Streptomyces griseus	5.26

Table 4.6: The percentage of lamb mouth samples containing each bacterial species (*n*=6)

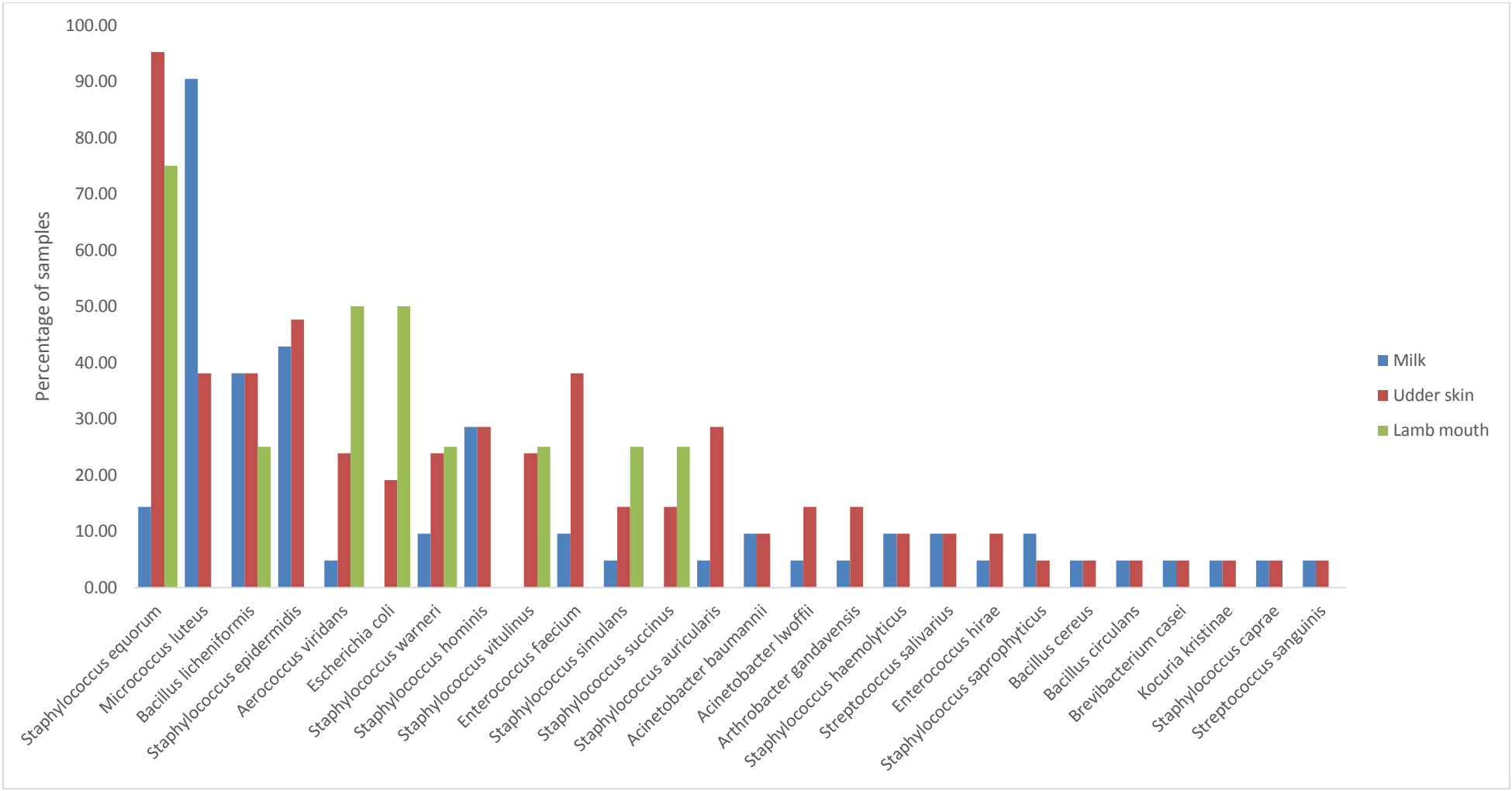
Bacterial Species	Lambs Mouth
Staphylococcus equorum	50
Streptococcus entericus	50
Aerococcus viridans	33.33
Enterococcus faecium	33.33
Escherichia coli	33.33
Bacillus licheniformis	16.67
Corynebacterium urealyticum	16.67
Enterococcus faecalis	16.67
Enterococcus hirae	16.67
Proteus vulgaris	16.67
Staphylococcus simulans	16.67
Staphylococcus succinus	16.67
Staphylococcus vitulinus	16.67
Staphylococcus warneri	16.67

Table 4.7: The percentage of skin (pre and post lambing) samples containing each bacterial species (*n*=6, *n*=102 respectively)

Bacterial Species	Skin pre-lamb	Skin post-lamb
Staphylococcus equorum	100	71.4
Bacillus licheniformis	12.5	42.9
Staphylococcus epidermidis	0	47.6
Micrococcus luteus	0	42.9
Staphylococcus auricularis	12.5	28.6
Enterococcus faecium	0	38.1
Staphylococcus vitulinus	12.5	19
Staphylococcus hominis	0	28.6
Aerococcus viridans	0	23.8
Staphylococcus aureus	0	23.8
Staphylococcus warneri	0	23.8
Bacillus pumilus	0	19
Escherichia coli	0	19
Corynebacterium casei	12.5	4.8
Acinetobacter lwoffii	0	14.3
Arthrobacter gandavensis	0	14.3
Staphylococcus simulans	0	14.3
Staphylococcus succinus	0	14.3
Staphylococcus xylosus	0	14.3
Bacillus safensis	12.5	0
Lysinibacillus fusiformis	12.5	0
Proteus vulgaris	12.5	0

Bacterial Species	Skin pre-lamb	Skin post-lamb
Staphylococcus lentus	12.5	0
Acinetobacter baumannii	0	9.5
Acinetobacter sp	0	9.5
Acinetobacter towneri	0	9.5
Enterococcus hirae	0	9.5
Kocuria carniphila	0	9.5
Solibacillus silvestris	0	9.5
Staphylococcus chromogenes	0	9.5
Staphylococcus haemolyticus	0	9.5
Streptococcus anginosus	0	9.5
Streptococcus salivarius	0	9.5
Acinetobacter johnsonii	0	4.8
Arthrobacter citreus	0	4.8
Bacillus cereus	0	4.8
Bacillus circulans	0	4.8
Bacillus megaterium	0	4.8
Bacillus weihenstephanensis	0	4.8
Brevibacterium casei	0	4.8
Corynebacterium freneyi	0	4.8
Enterococcus casseliflavus	0	4.8
Enterococcus mundtii	0	4.8
Granulicatella adiacens	0	4.8
Halomonas elongata	0	4.8
Kocuria kristinae	0	4.8
Kocuria rosea	0	4.8
Lactobacillus vini	0	4.8
Oerskovia turbata	0	4.8
Paenibacillus amylolyticus	0	4.8
Rhodococcus fascians	0	4.8
Rothia amarae	0	4.8
Staphylococcus caprae	0	4.8
Staphylococcus saprophyticus	0	4.8
Staphylococcus schleiferi	0	4.8
Staphylococcus sciuri	0	4.8
Streptococcus pluranimalium	0	4.8
Streptococcus salivarius_ssp_thermophilus	0	4.8
Streptococcus sanguinis	0	4.8
Streptococcus uberis	0	4.8

Figure 4.6: Percentage of ewes with samples taken by species isolated from lamb mouths, ewe udder skin and ewe milk.



4.4.3. Transmission and persistence analysis

Composite correlation indices were used to suggest potential transmission pathways and events of persistence. These thresholds are not absolute, as indicated in Chapter 3 and provide evidence for isolates and pathways of interest. These pathways should be tested further using established strain typing techniques. Although the CCI estimates are extremely high for extracted technical replicates, these cannot be used for isolates from different ewes and/or locations as they would result in missed transmission events as these are technical rather than biological replicates. The variability between biological replicates has not been shown in the literature; however it is likely that composite correlation indices would be substantially lower than the technical replicate estimates.

Lamb mouth and udder skin

Due to only having lamb mouth samples for the first observation, transmission events following on from this observation involving lamb mouths could not be investigated in this study. However, the presence of the same strain types in lamb mouths and on udder skin could suggest a potential transmission pathway, particularly when taking into account differences in species present on udder skin pre and post lambing.

For example, *Escherichia coli* was found on the udder skin of a ewe at the first observation after lambing, at the same observation it was also isolated from the ewe's lamb's mouth. The two isolates identified from the ewe's udder skin had CCI estimates of between 0.63 and 0.82 in comparison to lamb mouth isolates (Table 4.8). Although there are no technical replicate estimates for *Escherichia coli* specifically to define CCI cut-offs, 0.82 fits within the ranges of CCI estimates reported in Chapter 3 for isolates of the same strain type in other species and genera.

Although these samples were taken at the same time point, this ewe had no *Escherichia coli* present on the udder skin pre-lambing.

Table 4.8: Composite correlation indices matrix for *Escherichia coli* isolates found on the udder skin and in lamb mouths for ewe G72 at the same observation (Visit 1).

7. Lamb mouth isolate *G72L1MCC*							1.00
6. Lamb mouth isolate *G72L1MCA*						1.00	0.95
5. Lamb mouth isolate *G72L1BDE*					1.00	0.75	0.76
4. Lamb mouth isolate *G72L1BDD*				1.00	0.77	0.92	0.92
3. Lamb mouth isolate *G72L1BDC*			1.00	0.92	0.72	0.95	0.94
2. Udder skin isolate *43533 S MC B*		1.00	0.81	0.81	0.68	0.80	0.82
1. Udder skin isolate *43533 S MC A*	1.00	0.81	0.73	0.78	0.63	0.75	0.77
	1	2	3	4	5	6	7

For the same ewe and lamb, *Enterococcus faecium* was isolated at the same observation (lambing). In addition, the CCI estimates for the udder skin isolate ranged from 0.66-0.82 in comparison to the lamb mouth isolates (Table 4.9). Although there are no technical replicate estimates for *Enterococcus faecium* specifically to define CCI cut-offs, the values (0.66-0.82) for the udder skin isolates compared to the lamb mouth isolates fit within the ranges (0.65-0.91) of CCI estimates found in Chapter 3 for isolates of the same strain type in other species and genera.

Table 4.9: Composite correlation indices matrix for *Enterococcus faecium* isolates from the udder skin and in lamb mouths for ewe G72 at the same observation (Visit 1).

5. Lamb mouth isolate *G72L2MCG*					1.00
4. Lamb mouth isolate *G72L2MCF*				1.00	0.97
3. Lamb mouth isolate *G72L2EDG*			1.00	0.98	0.97
2. Lamb mouth isolate *G72L1EDD*		1.00	0.78	0.77	0.77
1. Udder skin isolate *43533 S BD B*	1.00	0.66	0.78	0.76	0.82
	1	2	3	4	5

Skin before lambing to skin after lambing

When comparing *Staphylococcus equorum* (present in lamb mouth, ewe milk and udder skin swab samples) for the same ewe, the strain found in the ewe's lambs mouth was the same strain type on the udder skin pre-lambing, at the first observation post lambing and a subsequent observation (Table 4.10). The CCI estimates ranged from 0.63 to 0.78 in comparison to the lamb mouth isolate. Based on Chapter 3 this would indicate the lamb mouth strain is not the same as any of the strains isolated from the udder skin. However, the strain type present on the skin pre and post lambing had CCI estimates up to 0.93 which is above the cut-off point for

Staphylococcus equorum as found in chapter 3 indicating that these might be the same strain type. This was also the case for several other ewes, where a CCI score of >0.90 was identified for isolates taken from the skin pre and post lambing. An example of the *Staphylococcus equorum* isolates retrieved from skin pre and post lambing and the corresponding CCI estimates for one ewe are shown in Appendix 3 and 4.

Table 4.10: Composite correlation indices for *Staphylococcus equorum* isolates on udder skin (pre and post lambing) and lamb mouth for ewe W15.

Record date	Visit	Sample type	Analyte	CCI estimate compared to Lamb mouth isolate 1	CCI estimate compared to Skin Post-lambing isolate 3
18-Apr-11	1	Skin Pre-lambing	*W15MCH*	0.63	0.74
18-Apr-11	1	Skin Pre-lambing	*W15MCE*	0.75	0.93
18-Apr-11	1	Skin Pre-lambing	*W15BDB*	0.77	0.90
18-Apr-11	1	Skin Pre-lambing	*W15BDA*	0.69	0.92
18-Apr-11	1	Skin Pre-lambing	*W15MCA*	0.74	0.69
18-Apr-11	1	Lamb mouth	*W15L1MCH*	1.00	0.78
21-Apr-11	2	Skin Post-lambing	*43953 S MC C*	0.70	0.75
21-Apr-11	2	Skin Post-lambing	*43953 S MC B*	0.72	0.88
21-Apr-11	2	Skin Post-lambing	*43953 S MC A*	0.78	1.00
19-May-11	3	Skin Post-lambing	*44851 S MC C*	0.70	0.84

Udder skin and milk

Udder skin to milk

A CCI estimate of 0.69 was identified for a pair of *Bacillus licheniformis* isolates from the udder skin pre-lambing and the milk (Table 4.11). However this same strain was not identified on the udder skin at the same observation it was isolated from the milk.

Table 4.11: Composite correlation indices for *Bacillus licheniformis* isolates from udder skin pre lambing, udder skin post lambing and lamb mouths for ewe G50.

Record date	Visit	Sample type	Analyte	CCI estimate compared to Milk isolate 1
Approx. 1 week pre lambing	1	Skin Pre lambing	*G50MCB*	0.69
07-Apr-11	2	Skin	*43526 S MC*	0.44
07-Apr-11	2	Milk	*43526 MC*	1.00

Based on a cut-off of >0.44 (as found in Chapter 3 for *Staphylococcus warneri*), the same strain types of *Staphylococcus epidermidis* were also found on the udder skin and in the milk at different time points (Table 4.12). For example, the same isolate was found on the udder skin on 21st April 2011 and in the milk on 19th May 2011 (CCI:0.62). In addition, another isolate in the milk isolated on 19th May 2011 was the same strain type as an udder skin isolate on the same date (CCI:0.60).

Table 4.12: Composite correlation indices matrix for *Staphylococcus epidermidis* isolates from udder skin and milk of the same ewe at different time points for ewe W75.

8. 19-May-11 (Visit 3) Skin Isolate *44660 S MC F*								1.00
7. 19-May-11 (Visit 3) Milk Isolate *44660 MC*							1.00	0.46
6. 19-May-11 (Visit 3) Skin Isolate *44660 S MC C*						1.00	0.44	0.66
5. 19-May-11 (Visit 3) Milk Isolate *44660 MC C*					1.00	0.52	0.67	0.53
4. 19-May-11 (Visit 3) Skin Isolate *44660 S MC A*				1.00	0.47	0.89	0.42	0.66
3. 19-May-11 (Visit 3) Milk Isolate *44660 MC D*			1.00	0.50	0.47	0.53	0.38	0.60
2. 19-May-11(Visit 3) Milk Isolate *44660 MC A*		1.00	0.32	0.24	0.22	0.21	0.27	0.29
1. 21-Apr-11 (Visit 2) Skin Isolate *43601 S BD A*	1.00	0.23	0.28	0.33	0.44	0.36	0.62	0.30
	1	2	3	4	5	6	7	8

The same was found for *Staphylococcus warneri* isolates. The same isolate was identified on the udder skin and in the milk on 10th March 2011 (Table 4.13 and 4.14).

Table 4.13: Composite correlation indices for *Staphylococcus warneri* isolates from udder skin post lambing and ewe milk for ewe OR15.

Record date	Visit	Sample type	Analyte	CCI compared to Milk isolate 2
10-Mar-11	1	Skin	*42284 S MC G*	0.10
10-Mar-11	1	Skin	*42529 S BD C*	0.51
10-Mar-11	1	Skin	*42284 S MC C*	0.17
10-Mar-11	1	Milk	*42529 BD E*	0.52
09-May-11	3	Milk	*44561 BD B*	1.00

Table 4.14: Full Composite correlation indices matrix for *Staphylococcus warneri* isolates from udder skin post lambing and ewe milk for ewe OR15.

5: 9-May-11 (Visit 3) Milk Isolate *44561 BD B*					1.00
4: 10-Mar-11(Visit 1) Milk Isolate *42529 BD E*				1.00	0.52
3: 10-Mar-11 (Visit 1) Skin Isolate *42529 S BD C*			1.00	0.42	0.51
2: 10-Mar-11 (Visit 1) Skin Isolate *42284 S MC G*		1.00	0.10	0.15	0.10
1: 10-Mar-11 (Visit 1) Skin Isolate *42284 S MC C*	1.00	0.60	0.13	0.15	0.17
	1	2	3	4	5

Milk to udder skin

Micrococcus luteus isolates of the same strain type were first identified in the milk and then on udder skin at a later observation for 2 ewes based on the 0.70 cut-off point for *Micrococcus luteus* (Table 4.15 and 4.16).

Table 4.15: Composite correlation indices for *Micrococcus luteus* isolates from udder skin post lambing and ewe milk for ewe W85.

Record date	Sample type	Analyte	CCI in comparison to Skin isolate 1
28-Mar-11	Milk	*43724 MC A*	0.80
19-May-11	Milk	*44714 BD A*	0.85
19-May-11	Milk	*44714 BD B*	0.78
19-May-11	Milk	*44905 MC A*	0.61
19-May-11	Skin	*44905 S MC B*	1.00

Table 4.16: Composite correlation indices for *Micrococcus luteus* isolates from udder skin post lambing and ewe milk for ewe OR75.

Record date	Milk/Skin	Analyte	CCI in comparison to Skin isolate 1
21-Mar-11	Milk	*42222 BD A*	0.60
21-Mar-11	Milk	*42222 BD B*	0.70
07-Apr-11	Milk	*43960 BD A*	0.62
07-Apr-11	Milk	*43960 BD B*	0.56
07-Apr-11	Milk	*43960 MC B*	0.54
07-Apr-11	Milk	*43724 MC A*	0.61
07-Apr-11	Skin	*43960 S BD B*	1.00

Persistence in milk over time

The same strain types were consistently isolated from the milk of the same ewes over time for *Bacillus licheniformis* (CCI: 0.8, Appendix 5), *Staphylococcus hominis* (CCI: 0.90, Appendix 6) and *Staphylococcus warneri (*CCI: 0.52, Table 4.13 and 4.14).

Skin to skin of other ewes

There were several examples where the same strain type was present on multiple ewes' udders over time. For example for *Staphylococcus aureus*, all but 1 isolate were isolated from the skin (Table 4.17). The milk isolate appeared to be very different from all other isolates. Aside from this isolate, there are 3 others that differ a great deal from all other *Staphylococcus aureus* isolates from the skin. This can also be viewed in a dendrogram more clearly (Figure 4.7).

With scores of between 0.01-0.45 (MALDI types 1-4) for very separated isolates, and up to 0.99 (MALDI type 5) for very similar isolates it appears that the majority of ewes (5/7) have the same strain type present on the udder skin (Table 4.17 and 4.18). These types were isolated over a period of 3 months from 8 ewes (Table 4.17). Although a cut-off point was not provided for *Staphylococcus aureus* in Chapter 3, the CCI estimates for technical replicates (Table 4.2) indicates that technical replicates can be as low as 0.95. Therefore we can assume that all pairs with CCI scores above this are indistinguishable by MALDI-ToF-MS. However, this cut-off is likely to be lower that the CCIs for the technical replicates, and could be similar to the cut-off for *Staphylococcus equorum* (0.88). Based on this cut-off, all but 4 isolates were the same strain type.

Table 4.17: Ewe ID, Record date, Analyte and assigned MALDI type number for all *Staphylococcus aureus* isolates found in the study from udder skin and ewe milk.

Ewe ID	Record date	Analyte	MALDI type
OR15	11-Apr-11	*44387 BD B*	2
OR75	07-Apr-11	*43960 MC D*	5
OR75	07-Apr-11	*43960 MC E*	5
OR75	07-Apr-11	*43960 MC G*	5
OR75	07-Apr-11	*43960 MC F*	5
OR75	07-Apr-11	*43960 MC A*	5
OR75	07-Apr-11	*43960 MC C*	5
OR75	07-Apr-11	*43960 MC B*	5
OR90	05-May-11	*45766 MC G*	5
OR95	05-May-11	*45742 MC C*	5
OR95	05-May-11	*45742 MC D*	4
W15	21-Apr-11	*43953 MC F*	5
W15	19-May-11	*44851 MC B*	5
W40	21-Apr-11	*44141 MC H*	5
W85	19-May-11	*44905 BD*	1
OR95	17-Mar-11	*42413 A MC**	3

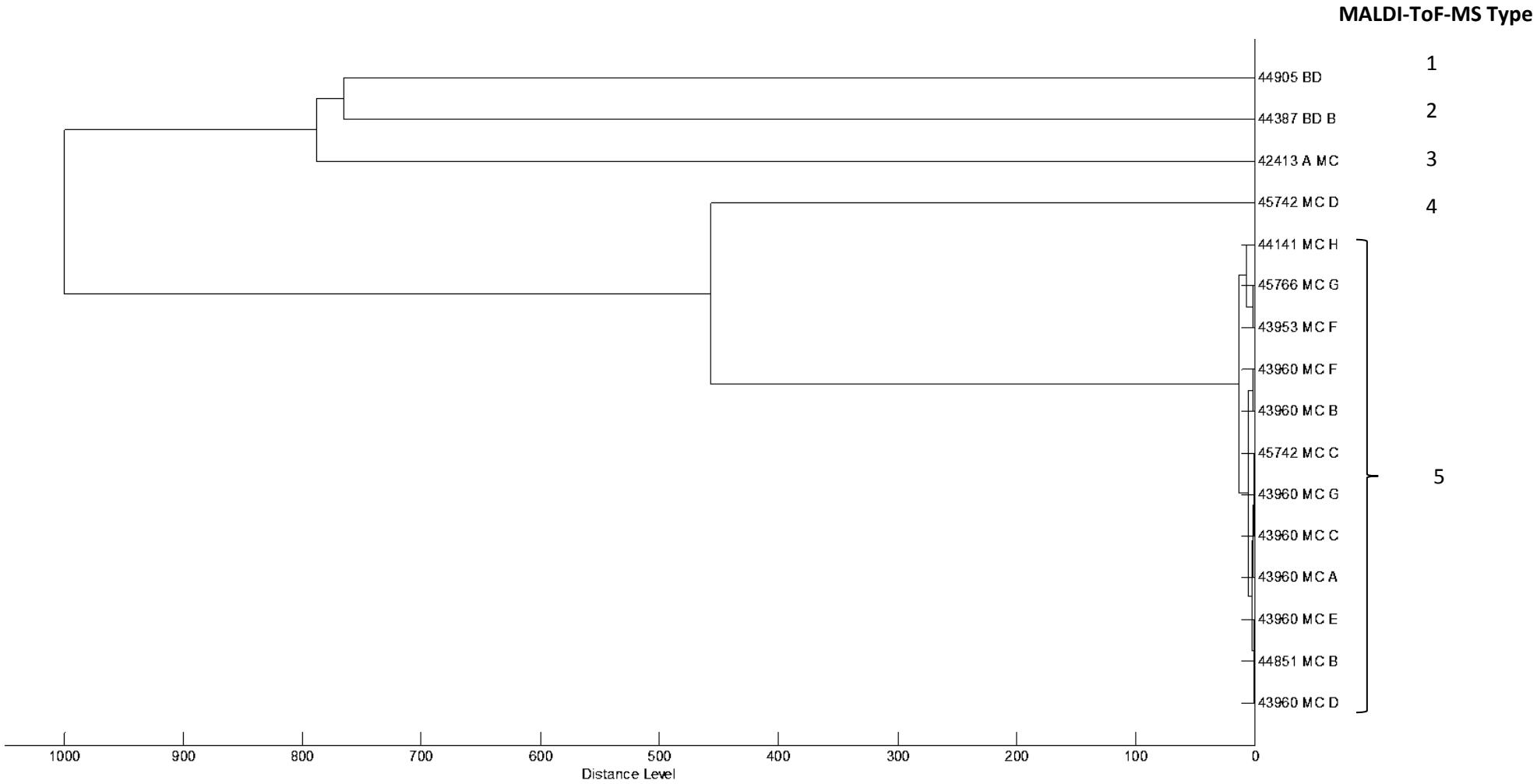
*- Isolate found in milk

Note: S has been removed from these analyte names

Table 4.18: Composite correlation indices matrix for all *Staphylococcus aureus* isolates found in the study (all isolates bar stared isolate is a skin isolate).

15: 45742 MC D															1.00
14: 45742 MC C														1.00	0.39
13: 44905 BD													1.00	0.08	0.00
12: 44851 MC B												1.00	0.04	0.85	0.49
11: 44387 BD B											1.00	0.20	0.00	0.21	0.00
10: 44141 MC H										1.00	0.16	0.88	0.05	0.69	0.44
9: 43960 MC G									1.00	0.69	0.17	0.86	0.04	0.93	0.37
8: 43960 MC F								1.00	0.78	0.91	0.19	0.90	0.03	0.73	0.45
7: 43960 MC E							1.00	0.96	0.74	0.97	0.17	0.94	0.04	0.73	0.45
6: 43960 MC D						1.00	0.94	0.94	0.90	0.89	0.21	0.93	0.05	0.87	0.44
5: 43960 MC C					1.00	0.72	0.60	0.60	0.75	0.57	0.24	0.66	0.00	0.73	0.01
4: 43960 MC B				1.00	0.64	0.95	0.99	0.95	0.77	0.96	0.22	0.92	0.05	0.75	0.45
3: 43960 MC A			1.00	0.65	0.02	0.72	0.61	0.60	0.74	0.58	0.01	0.67	0.06	0.74	0.01
2: 43953 MC F		1.00	0.66	0.91	0.65	0.88	0.91	0.87	0.79	0.91	0.18	0.88	0.08	0.78	0.44
*1: 42413 A MC**	1.00	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15

Figure 4.7: Biotyper 3.0 dendrogram for all *Staphylococcus aureus* isolates from the study and corresponding MALDI-ToF-MS Types.



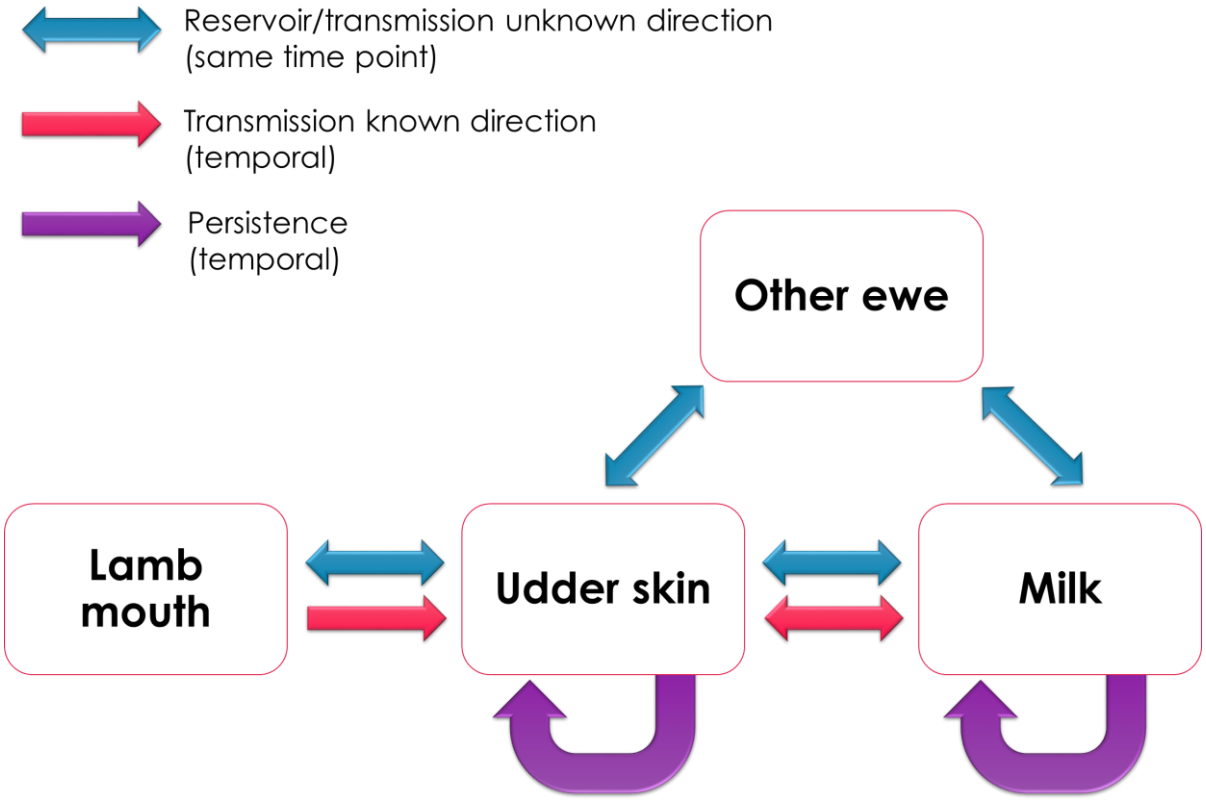
A summary of potential transmission events and/or persistence within the same ewe and/or her lambs were found for a variety of species (Table 4.19).

Table 4.19: Summary of potential transmission events and/or persistence within the same ewe and/or her lambs.

Transmission event within a ewe	Bacterial species
Skin to lamb mouth	*E.faecium*
Lamb mouth to skin	*E.faecium, E.coli*
Milk to skin	*M.luteus*
Skin to milk	*S.equorum, S.epidermidis, S.warneri*
Skin persistence	*S.equorum, S.aureus*
Milk persistence	*B.licheniformis, S.hominis, S.warneri*

Although the results provide evidence for potential transmission events and persistence only, there were many cases where the same strain types as defined by CCI cut-offs were found at the same time point in different environments, for example in the ewe milk and on udder skin at the same time point. In this instance, we would be unable to determine whether this was a transmission event and therefore it is defined as a reservoir, co-location and/or a transmission event of unknown direction. Identical strain types were found within samples from ewes over time indicating events of transmission where the locations changed, and persistence where the locations remained the same. A summary of the potential transmission events and persistence within and between ewes and lambs found in this study is summarised in Figure 4.8.

Figure 4.8: Summary of potential transmission events and persistence found in this study.



4.5. Discussion

The aim of this study was to identify potential transmission events and persistence of the same bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time using MALDI-ToF-MS.

In chapter 3, all isolates were all cultured on SBA and run through MALDI-ToF-MS using the direct smear method. To improve the success rate of isolating bacteria typically associated with mastitis and to ensure a wide range of bacteria were selected, a variety of selective media were used. This allowed the selection of all isolates from milk samples grown on SBA plates and up to 5 of each morphologically unique isolate from Edwards and MacConkey agar. In this study, despite ewes not having clinical mastitis, a wider variety of mastitis causing pathogens were isolated from ewe milk, udder skin and lamb mouths compared to Chapter 3 where few mastitis causing pathogens were identified. The selective media may have enhanced the growth of these species, allowing them to be identified more

easily. Future studies on transmission and reservoirs could utilise this approach to improve chances of isolating mastitis causing bacteria.

The use of a variety of agar for MALDI-ToF-MS has been shown before (Valentine *et al.* 2005). A core set of proteins appear to be consistent under different culture media conditions and using a variety of SBA plates (Valentine *et al.* 2005). However using the direct method on varying agar can also result in reduced scores (Anderson *et al.* 2012). To overcome the reduced scores due to varying media, extraction was necessary during this study. This study agrees with previous studies in that chemical extraction enhances bacterial identification compared to the direct smear method for anaerobic bacteria (Fournier *et al.* 2012, Khot *et al.* 2012).

Technical replicate CCI variation showed the consistency of these scores, particularly for *Streptococcus uberis* and *Streptococcus agalactiae* where 5/6 technical replicates had CCI estimates of 1.00 (the highest estimate possible). The CCI estimates for technical replicates of *Staphylococcus aureus* were lower, indicating this species has more spectral variability. These technical replicate CCI estimates, coupled with the CCI estimates for the same and different strain types highlighted in Chapter 3 indicate that there is low variability of spectra between technical replicates, but there is some spectral variability between isolates of the same strain type when comparing MALDI-ToF-MS to PFGE.

To the authors knowledge, the only mastitis-linked organism isolated from lamb mouths is *Mannheimia haemolytica* (Fragkou *et al.* 2011). In this study a variety of species were isolated including bacteria associated with mastitis (Table 4.5). This could indicate that the lamb mouth acts as a reservoir for mastitis causing bacteria. This is supported by studies on horizontal transmission of *Mannheimia* species between ewes via lambs sucking (Fragkou *et al.* 2011) and from the mouths of ewes and lambs to teat skin (Scott & Jones 1998).

The most frequently isolated species from ewe milk was *Bacillus licheniformis, Micrococcus luteus,* and coagulase-negative staphylococci. Coagulase-negative staphylococci species have been frequently isolated from bovine dairy herds (Gillespie *et al.* 2009) and in ewes without clinical mastitis (Pengov 2001). *Bacillus*

licheniformis has also been isolated from the milk from cows with mastitis (Nieminen *et al.* 2007, Salkinoja-Salonen *et al.* 1999).

As was expected, a large variety of bacterial species were isolated from the udder skin (Table 4.7). There are no previous studies on the characterisation of bacterial populations on the udder skin and teat ends of sheep. However, studies in cows have indicated a difference in populations between beef and dairy (Gill *et al.* 2006). The most frequently isolated bacteria present in cows differed in some cases from those present in this study, although this could also be due to the different methods used. However *Staphylococcal* species were isolated from cows (Gill *et al.* 2006) and the ewes in this study.

Twenty-six species out of a total of 75 (where the MALDI-ToF-MS confidence score was ≥1.7) were found in more than one location (lamb mouth, ewe udder skin and/or ewe milk). These species are of interest as they have the potential to be involved in the transmission pathways of mastitis.

Staphylococcus equorum has never been previously reported as isolated from lamb mouths. In the current study, *Staphylococcus equorum* was the most frequently isolated species from lamb mouths and udder skin and it was also frequently isolated from ewe milk although *Micrococcus luteus* was the most frequently isolated bacterial species from milk. Previous studies in cows (Vacheyrou *et al.* 2011) also frequently identified *Staphylococcus equorum* from the cow udder skin, although no studies have isolated *Staphylococcus equorum* from sheep skin.

The fact that some bacterial species were found in all three locations (udder skin, lamb mouths and ewe milk) and that some were only present in 2 of the 3 locations may give some indication as to their transmission pathways. For example, *Micrococcus luteus, Staphylococcus epidermidis, Staphylococcus hominis, Enterococcus faecium* and *Staphylococcus auricularis* were the most commonly isolated species from only udder skin and milk but were not isolated from lamb mouths (where *Escherichia coli* and *Staphylococcus succinus* were detected, for example). This could indicate the udder skin acts as a reservoir for these species rather than lamb mouths. This contributes new knowledge as there is no previous research on transmission pathways of these species in suckler sheep.

Interestingly, some bacterial species associated with mastitis were only found on udder skin after lambing (and therefore after suckling had occurred); suggesting lamb mouths play a vital role in transmission of these species. However, it must be noted that only 8 ewes were sampled pre-lambing and therefore the variety of bacterial species isolated from this type of sample may be limited in this study.

In this study, 43.7% of ewes had *Staphylococcus aureus* isolated from the udder skin after lambing, although *Staphylococcus aureus* was only isolated from the milk of 1 ewe at 1 time-point. Colonisation of the teat skin with *Staphylococcus aureus* is thought to play a major role in intramammary infections. In a study of 20 cows, quarters with teat skin colonised with *Staphylococcus aureus* were 4.5 times more likely to be diagnosed with intramammary infections attributed to *Staphylococcus aureus* than quarters where *Staphylococcus aureus* had not colonised the teat skin (da Costa *et al.* 2014). It is surprising that despite a large number of ewes having *Staphylococcus aureus* colonised on the udder skin throughout lactation, *Staphylococcus aureus* was only isolated from one milk sample from one ewe at one time point, and MALDI-ToF-MS analysis indicated this to be a very different strain from the rest of the isolates found in the study. This further highlights the multifactorial nature of mastitis; the microbial environment of the ewe is just one factor in a ewe's likelihood to develop mastitis.

This is the first study to utilise MALDI-ToF-MS for the analysis of potential transmission events and persistence of a bacterial strain type. Combining CCI estimates for technical replicates, along with the PFGE based CCI cut-offs for a variety of species in Chapter 3 provided potential transmission events between and within environments over time. Most notably, *Escherichia coli*, a major mastitis pathogen was isolated from lamb mouths and udder skin at the first observation after lambing but not in ewe milk or udder skin pre-lambing. In addition, the CCI estimates for these isolates indicated that they were the same strain type, further supporting the possibility of a potential transmission event. This is the first time *Escherichia coli* has been isolated from lamb mouths, or been implicated in transmission using strain typing from lamb mouths to udder skin.

The same strain types, as indicated by MALDI-ToF-MS, were found at different observations for a variety of coagulase-negative staphylococci to and from ewe milk

and udder skin. The same strain types of *Staphylococcus aureus* have been isolated from milk and body samples (particularly hock skin) in cows previously (Capurro *et al.* 2010), although no reference was made to which direction this transmission may have occurred in, and the study only referred to a possible reservoir of *Staphylococcus aureus*.

In addition, persistence of the same strain types were found in the milk for a variety of species over the three observations (over several months). Although this could also be due to re-colonisation with the same strain, the number of ewes in which this occurred makes it more likely that they were persistent colonisations. Persistence of *Staphylococcus aureus* (Anderson & Lyman 2006) for up to 13 years in dairy farms has been shown using PFGE, as well as persistence of the same enterobacterial isolates in cows using DNA fingerprinting (Bradley & Green 2000). However persistence of *Bacillus licheniformis, Staphylococus hominis* and *Staphylococcus warneri* has previously never been shown.

Limitations to this work include only having 3 sampling points over a 10 week period. In order for 'actual' rather than 'potential' transmission events to be identified, shorter sampling time points would be necessary. In addition, MALDI-ToF-MS has been compared with PFGE, and although they correspond, single nucleotide polymorphisms (SNPs) or whole genome sequencing would ideally be used to confirm transmission events and have been for several species (Chen *et al.* 2007, Knetsch *et al.* 2014, Scaltriti *et al.* 2015, Snyder *et al.* 2013, Stucki *et al.* 2014).

In this study, isolates were picked from the udder skin based on morphology in order to try and select isolates that may have been present in a variety of environments. The morphology of isolates of the same species between different environments can differ, and therefore as many isolates as possible should be picked rather than convenience selecting those which look most likely to be involved in transmission. Despite this, the aim of the study was to utilise MALDI-ToF-MS in selecting potential transmission events rather than all transmission events, and therefore these methods were acceptable.

Future work could involve comparing these transmission events to somatic cell counts to see whether somatic cell count (indicating an immune response) increases after a potential transmission event.

4.6. Conclusion

A variety of species were isolated from lamb mouths, ewe udder skin and ewe milk that have not previously been isolated from these environments, most notably the presence of *Escherichia coli* in lamb mouths.

Potential transmission events and persistence of the same bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time were identified for the first time in suckler sheep using MALDI-ToF-MS for a variety of bacterial species.

Chapter 5. Are udder and teat conformation heritable traits that affect mastitis in Texel sheep?

5.1. Introduction

The average prevalence of clinical mastitis in sheep is <5% (Chapter 2). However, the prevalence of subclinical mastitis is likely to be much higher, ranging from 10-50% in small ruminants (Bergonier & Berthelot 2003).

Using parallels with cattle, it was estimated that mastitis was likely to cost the Texel sheep breed alone approximately £2.7 million per annum (Conington *et al.* 2008). There are many negative economic costs associated with mastitis including treatment, poor lamb growth rate, ewe replacement and carcass disposal costs (Huntley *et al.* 2012).

Mastitis, although caused by bacteria, is likely to be influenced by both genetic and environmental factors. Over the past few decades, research on the genetic element of mastitis in cattle has accumulated, however, a previous report indicated that there was a dearth of information on the causes of mastitis and the heritable traits that are likely to be associated with udder health in ewes (Conington *et al.* 2008).

Two studies have shown an association between genetic and phenotypic factors (specifically udder conformation) and mastitis in dairy ewes (Casu *et al.* 2010, Legarra & Ugarte 2005). In addition, poor udder and teat conformation, scored using an amended version of a published protocol (Casu *et al.* 2006), were associated with decreased lamb growth rate, increased levels of subclinical mastitis (measured by somatic cell count) (Huntley *et al.* 2012), and the occurrence of traumatic teat lesions (caused during suckling) (Cooper *et al.* 2013).

Low lamb weight was associated with high somatic cell count, an incident case of a teat lesion, sub-optimal teat position, rearing in more than one lamb and ewe age. High udder half somatic cell count (indicative of sub clinical mastitis) was associated with pendulous udders, and large cross-sectional area of the teats (Huntley *et al.* 2012). Traumatic teat lesions were associated with heavier total litter weight, body

condition score, udder skin condition, sub-optimal teat position and udder depth (Cooper *et al.* 2013).

The lowest somatic cell count, fewest traumatic teat lesions and greatest lamb growth rate was associated with teat position '5', where the teats are at the 4 and 8 o'clock position. This is in direct contrast to studies of dairy ewes where position 1 is thought to be the optimum, and highlights the potential pitfalls of comparing results from analyses of dairy and non-dairy ewes.

Improving ewe resilience to mastitis through selective breeding of heritable traits could ultimately reduce mastitis. However, the traits in question would need to be repeatable within and across lactations in order to be useful. Previous studies have indicated some udder conformation traits (in particular teat placement) have high repeatability within (de la Fuente *et al.* 2011, Fernández *et al.* 1995) and across lactation (de la Fuente *et al.* 2011) whilst others relating to udder size and therefore milk yield were affected by lactation and flock (Serrano *et al.* 2002). These traits also had lower heritability (Serrano *et al.* 2002). The repeatability and heritability of udder conformation traits in suckler ewes in England has not been explored.

5.2. Aims

The aims of this project were to record udder conformation scores of ewes in mid-lactation, along with cases of chronic clinical mastitis (detected by palpation of mammary gland abscesses), offspring and pedigree data in order to:

1. Assess whether udder and teat conformation were heritable traits; and

2. Investigate whether udder and teat conformation were phenotypically associated with higher levels of chronic mastitis.

The results of this project could ultimately lead to Estimated Breeding Values (EBV) for some udder and teat conformation traits, which could provide an objective way for Texel society members to assess the genetic potential of rams selected for breeding and female replacements in terms of mastitis resilience. This could eventually contribute to a reduction in mastitis, thus improving ewe and lamb welfare and increasing the net return of suckler sheep.

5.3. Methods

5.3.1. Flock & Ewe Selection

Ten performance recorded pedigree Texel flocks with individual ewe identification were convenience selected based on recommendations and farmer interest. Flocks included representatives from England, Scotland and Wales (Figure 5.1).

Figure 5.1: A map of study flock locations.

5.3.2. Data collection

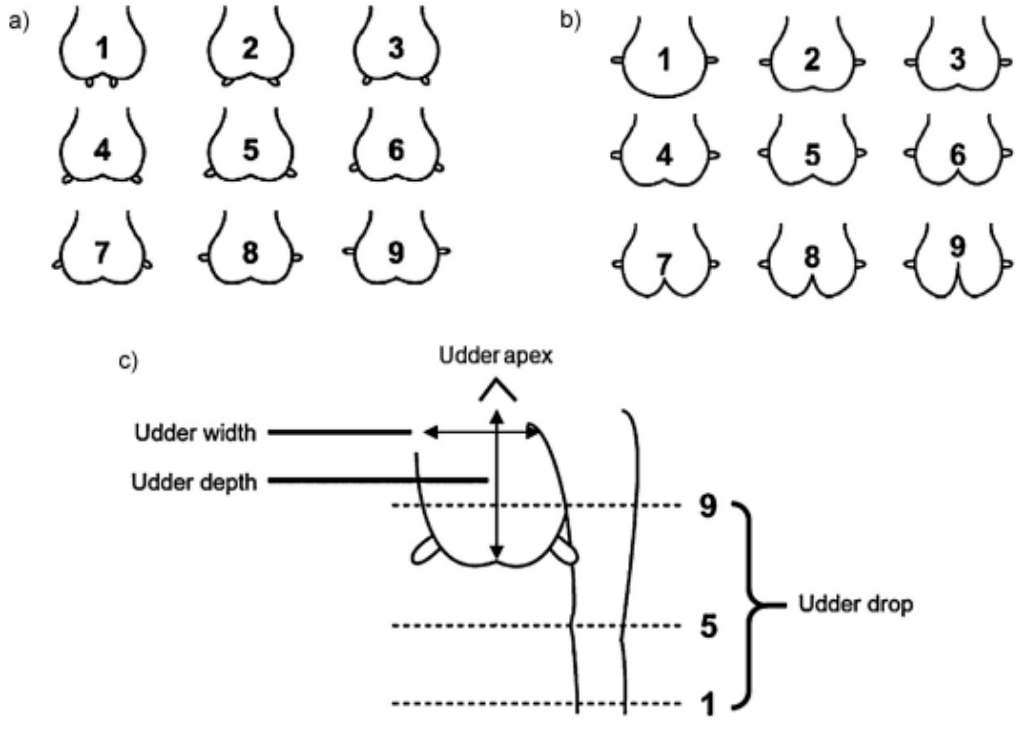
All Texel ewes from each flock were included in the study, which took place between 2012 and 2014. Two flocks were visited over several years. All ewes lambed between January and May of each year. Data collection occurred mid-lactation and included 10 udder and teat conformation traits, and individual ewe and lamb IDs.

Variable definitions

Udder traits

All ewes were scored for 10 udder and teat traits using a combination of linear scores, measurements, udder palpation and visual examination. Linear scores were used to characterise teat placement, udder drop, and the degree of separation of the udder halves as described previously (Casu *et al.* 2006, Marie-Etancelin *et al.* 2005) (Figure 5.2). A tape measure was used to record udder width from the rear at the widest point and the left teat length only because the initial study indicated a strong positive correlation between left and right udder teat length and width. Udder masses were detected by manual palpation of each udder half, and teat lesions were observed by visual inspection. All measurements were performed by two researchers and data recorded on custom-designed data recording sheets.

Figure 5.2: Scoring methods for a) teat placement, b) degree of separation and c) udder drop and udder width.



Binary variables

Chronic mastitis was defined as a palpable mass in either udder half.

Teat lesions (left and/or right) were defined as any lesion present on the teat at the observation. These could include bites, tears and grazes, which tended to be attributed to suckling; or spots, warts and/or proliferative scabs on the teat.

5.3.3. Data summary and analyses

All data were entered into Microsoft Excel spreadsheets. Individual ewe data were matched to flock book numbers via BASCO or by the farmers. Flock level pedigree data including sire and dam IDs were obtained from the BASCO database (BASCO). Data were moved into Microsoft Access. Queries were used to select and link data from Microsoft Access for statistical analysis. Ewe age in years at the time of observation was calculated as the difference between the date of birth and the observation date. Number of days in milk was calculated as the difference between the ewes' lambing date and the observation date.

Measures of dispersion and central tendency were used to investigate the data (R Core Team 2013). Obvious errors were corrected where possible.

5.3.4. Univariate variance component estimation for udder traits

Data and pedigree

The udder trait data file contained 968 records with BASCO identifier and associated pedigree information. Some data edits were performed sequentially to remove:

1. All Blue Texels (leaving 953 records);
2. Records with either conformation scoring or lambing date missing (876);
3. Records with unknown sire (833); and
4. Records for one year old ewes (817).

The 817 udder trait records were on 740 ewes; 665, 73 or 2 having been recorded on one, two or three occasions.

All pedigree records (associated with estimated breeding value reports) for each recorded flock were extracted from the BASCO database and these were concatenated to provide an overall pedigree file. The 817 ewe records were matched to these pedigree data. The resulting pedigree contained the recorded animals plus all known ancestors. Pruning was performed to remove redundant members of the pedigree leaving a 2270 animal pedigree for inclusion in the animal model analyses. There were 188 sires and maternal grandsires within this pedigree (i.e. the records used to create the sire-maternal grandsire relationship matrix for sire model analyses). Recorded ewes were the offspring of 145 sires with an average of 5.6 records associated with their offspring but 39 had only a single recorded offspring in the data set excluding repeated records on ewes.

The recorded traits were either objective measurements on a continuous scale or subjective observations with either two or several categories. Those traits recorded on a continuous scale or with many categories were treated as if they were normally distributed, and are referred to as such in this document. Traits with only two categories (referred to variously as binary, or binomial or categorical in this document) were treated as binomial data.

A data set that included only one record per ewe was summarised, the latest observation on any ewe was retained to create this set. Means, standard deviations and number of missing records of the normally distributed traits are given in Table 5.1. The incidence of the different phenotypic observations for the binary traits retained for analysis is in Table 5.2. The Woolly Udder binary trait was dropped due to its low incidence (only 16 cases out of 817 records), while the left and right chronic mastitis traits were dropped because it could be difficult to differentiate between udder halves in the field.

Table 5.1: Summary of traits assumed to be normally distributed from the single observation per ewe data set.

	Mean	Standard deviation	Missing
Teat Placement	5.87	1.16	4
Udder Drop	6.93	0.86	4
Degree of Separation	3.23	1.38	54
Udder Width	13.45	2.09	5
Left Teat Length	2.50	0.44	4

Table 5.2: Incidence for binary traits in the single observation per ewe data set.

	No	Yes	P(x=1)	Missing
Chronic Mastitis	516	224	0.30	0
Left Teat Lesion	652	88	0.12	0
Right Teat Lesion	639	101	0.14	0
Any Teat Lesion	601	139	0.20	0

Fixed effects

The primary fixed effects considered were:

- A flock-by-scoring date (FSD) factor,
- The ewe age (EA) factor has 5 levels (2,3,4,5, and >5 years old); and
- A linear regression on days in milk (DIM).

In addition, some information on litter size was available. For records taken subsequent to the preliminary analyses, the number of lambs born was recorded. An approximation to this could also be made by counting the number of registered offspring of a ewe in the BASCO database, and this was used where the actual number of lambs born was not available. There were still a number of ewe records where no litter size information was available, and so litter size was considered in a series of separate analyses using a subset of the overall data. The two types of litter size record were fitted within the source of the record; such that there was a level for single and multiple births within each of 'observed' and 'BASCO-derived' (Table 5.3).

Table 5.3: Summary of litter size information used in analyses.

Source	Birth type	Data set (ewe records)	
		Single*	**All**
Observed	Single	123	124
	Multiple	127	128
BASCO-derived	Single	189	234
	Multiple	274	301
No litter size information		27	30
Total		740	817

*Latest observation retained on each ewe. The subset of these records with litter size information was used in the litter size analyses.

Data sets

For the different analyses, three data sets were used:

- all 817 records (when permanent environmental effects were considered to account for repeated measures);
- 740 single records per ewe, retaining the latest observation for each ewe, and
- 713 single records per ewe which also had litter size information (for use in analyses considering the impact of litter size).

Analyses

Univariate quantitative genetic analyses were performed using individual animal models (equation 1) and sire models (for binomial traits, equation 3). Repeated records were considered in individual animal model analyses including permanent environmental effects associated with ewes, equation 2.

When permanent environmental effects were excluded, the data used was the latest record on ewes recorded. This data set contained 740 records of 740 ewes rather than the 817 in the full data set.

The Bayesian Integrated Nested Laplace Approximation (INLA) method Rue *et al.* (2009) as implemented in the inla R package (http://r-inla.org) was used for both the animal and sire model analyses (Rue *et al.* 2009).

Comparison of fixed effect models

Quantitative genetic analysis was carried out for each trait with various combinations of the three primary fixed effects. The individual animal model (equation 1) was used for the normally distributed traits and the sire model (equation 3) for binary traits. For any trait, the model with the lowest Deviance Information Criterion (DIC) (Spiegelhalter *et al.* 2002) was selected for use in all subsequent analyses of that trait.

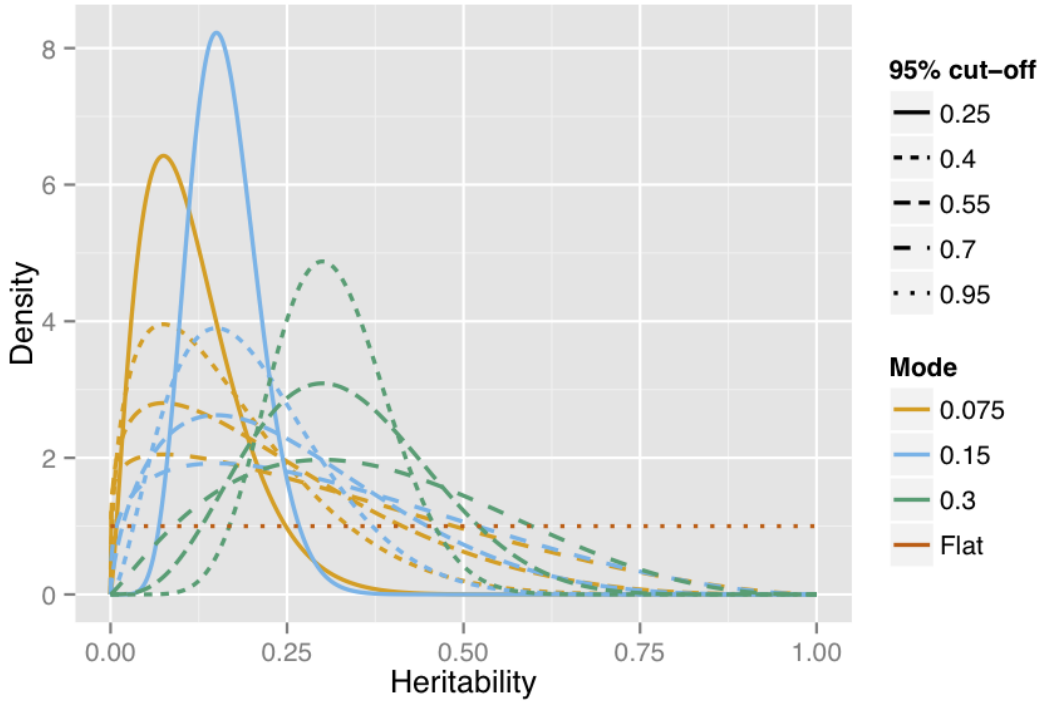
Inclusion of litter size

The litter size variable had four levels; single or multiple births for each of two data sources ('recorded' or 'BASCO-derived'). The subset of single ewe records with litter size information was analysed both including and excluding litter size category as an additional fixed effect in the model. Either an individual animal model (equation 1) or a sire model (equation 3) was used depending on the nature of the trait.

Sensitivity to priors

Bayesian analysis utilises prior information. The priors used in the main analyses are described later in this section. When there is sufficient information from the data the influence of the prior information upon the analysis results is negligible. In order to assess the sensitivity of our analyses to the chosen priors, analyses were repeated with various priors defined with respect to the heritability: the priors varied in terms of the mode and the width (and hence informativeness) of the prior distribution of the heritability. The range of prior distributions considered for the heritability is shown in Figure 5.3. The sensitivity analyses used the individual animal model for the normally distributed traits and the sire model for the binary traits. Repeated records were excluded from these analyses.

Figure 5.3: Prior distributions of the heritability considered in sensitivity analyses. The prior distribution was defined in terms of the mode and the 95% cut-off point of the heritability.



The individual animal model

The following individual animal models were considered:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{e} \quad (1)$$

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Za} + \mathbf{Zu} + \mathbf{e} \quad (2)$$

Where:

y is a vector of phenotypic observations;

b is a vector of fixed effects as selected from the analyses in section 5.3.4, analyses;

a is a vector of random animal effects;

u is a vector of random permanent environmental effects associated with ewes;

X and **Z** are design matrices relating observations to fixed effect levels or animals, respectively; and

e is a vector of random residual effects.

The variance of **a** is $var(\boldsymbol{a}) = \mathbf{A}\sigma_a^2$, where **A** is the numerator relationship matrix; the variance of **u** is $var(\boldsymbol{u}) = \mathbf{I}\sigma_{pe}^2$ and the variance of **e** is $var(\boldsymbol{e}) = \mathbf{I}\sigma_e^2$.

The heritability is $h^2 = \frac{\sigma_a^2}{\sigma_p^2}$, where the phenotypic variance is $\sigma_p^2 = \sigma_a^2 + \sigma_e^2$ without permanent environmental effects and $\sigma_p^2 = \sigma_a^2 + \sigma_{pe}^2 + \sigma_e^2$ for analyses including permanent environmental effects. The repeatability is $r = \frac{\sigma_a^2 + \sigma_{pe}^2}{\sigma_p^2}$.

For the binomial traits, the analysis was carried out using a binomial generalised linear mixed model (GLMM) with a logit link function. In these analyses, the residual variance has a fixed value, $\sigma_e^2 = \frac{\pi^2}{3}$.

The sire model

There is evidence that for categorical traits, particularly with non-intermediate frequencies, sire models provide better estimates of variance components than the individual animal model. To this end, a sire model with relationships was also considered for the binomial traits.

The data set used excluded repeated records, using the same 740 ewe records as in the non-repeatability individual animal model analyses.

The following sire model was used:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Zs} + \mathbf{e} \qquad (3)$$

Where:

y is a vector of phenotypic observations;

b is a vector of fixed effects as selected from the analyses in section 5.3.4, analyses;

s is a vector of random sire effects;

X and **Z** are design matrices relating observations to fixed effect levels or sires, respectively; and

e is a vector of random residual effects

The variance of **s** is $var(\mathbf{s}) = \mathbf{A_s}\sigma_s^2$ (where $\sigma_s^2 = 0.25\sigma_a^2$), where $\mathbf{A}_s$ is the sire-maternal grandsire numerator relationship matrix pertaining to 145 sires or recorded animals and their ancestors (a 188 animal sire-maternal grandsire pedigree).

A binomial GLMM with logit link function was used and the heritability was estimated as $h^2 = 4 \times \frac{\sigma_s^2}{\sigma_s^2 + \frac{\pi^2}{3}}$.

Priors

Prior distributions for heritabilities were generated. A beta distribution was assumed with a mode of 0.15 and in which 95% of the distribution fell below a heritability of 0.7. The same prior distribution of heritability was assumed for all traits and priors for the variance components were derived for each trait as follows:

Continuous traits

The heritability of a trait is $h^2 = \frac{\sigma_a^2}{\sigma_a^2 + \sigma_e^2} = \frac{\sigma_a^2}{\sigma_p^2}$ where σ_a^2, σ_e^2 and σ_p^2 are the additive direct genetic, residual and phenotypic variances for the trait. The heritability relates to the additive genetic and residual precisions thus: $\tau_a = \frac{1}{\sigma_a^2} = \frac{1}{h^2\sigma_p^2}$ and $\tau_e = \frac{1}{\sigma_e^2} = \frac{1}{(1-h^2)\sigma_p^2}$. Random samples from the prior distribution of the heritability may be converted into compatible random samples of τ_a and τ_e assuming that $\sigma_p^2 = 1$. Gamma distributions were then fitted to these samples. The rate parameter of the gamma distribution generated was then scaled by an estimate of $\sigma_p^2$to give priors specific to each continuous trait. The residual mean squares from multiple linear regression analyses fitting FSD, DIM and EA were used to approximate σ_p^2 for each continuous trait.

Binomial traits

The binomial traits were subject to a generalised linear mixed model with a logit link. Under the logit link function, the residual variance is fixed at $\sigma_e^2 = \frac{\pi^2}{3}$. Rearranging the equation for the heritability, the additive genetic and sire precisions

are $\tau_a = \frac{1-h^2}{h^2\sigma_e^2}$ and $\tau_s = \frac{4-h^2}{h^2\sigma_e^2}$, respectively. As above, the prior distributions for τ_a and τ_s were derived from a random sample from the prior distribution of the heritability. Rather than fitting a gamma distribution the R 'logspline' procedure (Kooperberg 2013) was used to approximate the distribution taking account of truncation, for the sire model $\tau_s \geq \frac{9}{\pi^2}$, to give a more realistic prior distribution.

Repeated records

To take some account of the presence of repeated observations on ewes, a repeatability model was considered. This requires the estimation of a permanent environmental variance component, σ_{pe}^2. In this model $\sigma_p^2 = \sigma_a^2 + \sigma_{pe}^2 + \sigma_e^2$ and the repeatability, $r = \frac{\sigma_a^2 + \sigma_{pe}^2}{\sigma_p^2}$. The methods described above to generate priors for continuous and binomial traits were extended to produce consistent priors for τ_a, τ_{pe} and τ_e.

5.3.5. Multivariable logistic regression for the association between udder traits and chronic mastitis

Ewes with duplicate records and/or no BASCO IDs (and therefore no BASCO/pedigree data) were deleted prior to modelling.

A single level multivariable binomial logistic regression model was constructed using R, with chronic mastitis of either udder half being the outcome variable. The variable days in milk and ewe age were forced into the model. The model took the form shown in equation 4:

$$g(Y) = \beta_0 + \sum \beta_m x_m \quad (4)$$

… where g is the log-link function, *Y* is the categorical outcome variable (whether the ewe had a lump in either udder half), β_0 is the intercept, and β_m the regression coefficients (expressing effects of the included predictor variables x_m). The independent variables were tested in the model using manual forward stepwise selection process. Significance was set at 0.05. Odds ratios were determined as exp(β) and the 95% confidence intervals for exp(β) were calculated (exp(β) ± 1.96 x standard error (SE)).

5.4. Results

5.4.1. Descriptive analyses

The average flock size across the 10 flocks in the study was 92 ewes (range 34-165). There were 873 ewes in the study, of which two year olds were the most frequent (Figure 5.4 A). Body condition score ranged from 1-4.5 with the majority of ewes (190 of 313 ewes scored) being a score of 2.5-3 (Figure 5.4 B). Ewes were scored on average at 65 days in lactation (range 5-123 days, Figure 5.5). Very few ewes were scored in the first 3 weeks of lactation.

Figure 5.4: Distribution of A) ewe age and B) Body condition score.

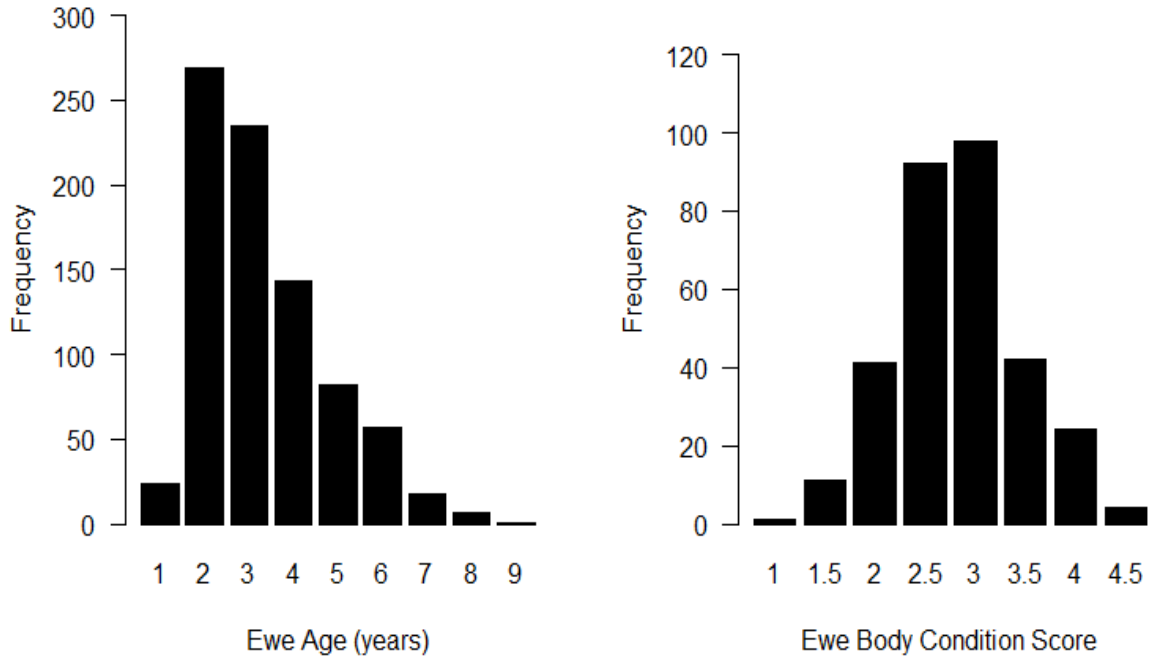
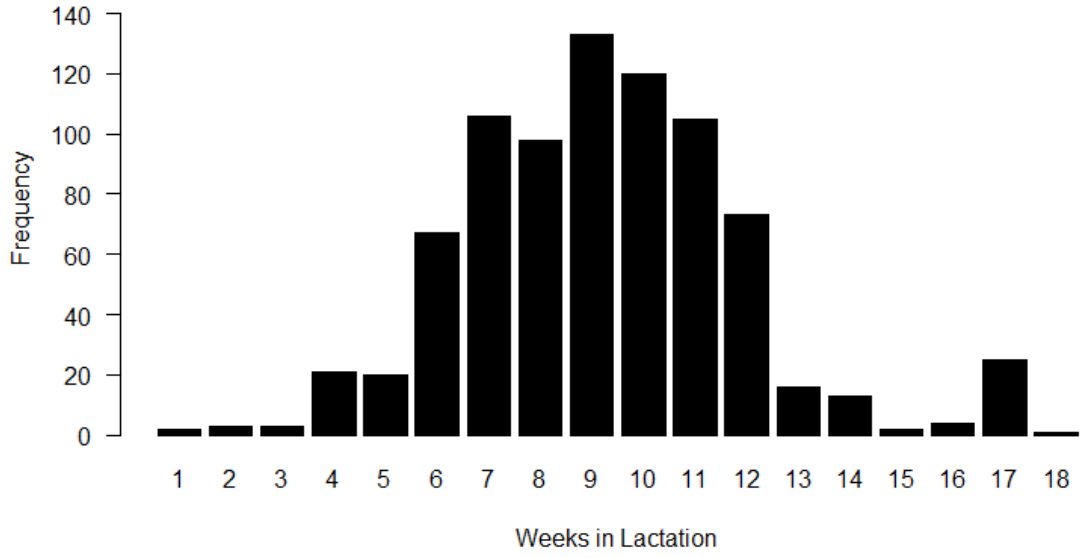


Figure 5.5: Distribution of weeks in milk when scored.



Two methods of litter size were included in the study to minimise missing data: Method 1-number of lambs per ewe recorded by researcher (believed to be more accurate) and method 2- number of lambs per ewe obtained through BASCO (increased number of records). The ewes included in the initial pilot study did not have litter size recorded by a researcher; this variable was added later during the full study hence the lower frequencies for method 1. On average, the litter size for researcher-recorded records were 1.59 lambs per ewe, and 1.66 lambs per ewe for records obtained through BASCO (Table 5.4).

Table 5.4: The number of ewes included and excluded in litter size analyses and the average litter size per ewe for two methods.

	Recorded	BASCO
Number of ewes with litter size data	287	765
Number of ewes with missing litter size data	586	108
Average litter size per ewe	1.59	1.66

From this point on in the analyses, distributions are based on record rather than ewe due to repeated measurements over multiple years for some ewes. There were 968 observations for 873 ewes.

Teat placement and udder drop scored were normally distributed around scores of 6 (range 2-9) and 7 (range 1-8) respectively whereas degree of separation was positively skewed around score 7 (range 3-9) (Figure 5.6).

Due to the consistency and correlation between left teat length, left teat width, right teat length and right teat width in the pilot study, the only teat measurement included in the full study was left teat length (Figure 5.7 A). Udder width was negatively skewed around 14cm (range 5-20cm) (Figure 5.7 B). This variable in particular has been linked in the literature to point of lactation in which the ewe was scored. The summary of traits is shown in Table 5.5.

Table 5.5: Summary of continuous traits assumed to be normally distributed.

	Mean	Standard deviation	Missing
Teat Placement	5.85	1.15	6
Udder Drop	6.92	0.85	6
Degree of Separation	3.18	1.37	63
Udder Width	13.47	2.05	7
Left Teat Length	2.49	0.43	6

Alongside teat and udder conformation variables, ewe udder half health and teat lesion presence were also recorded (Table 5.6).

Table 5.6: Incidence of binary traits.

	No	Yes	P(x=1)	Missing
Chronic mastitis	573	242	0.30	2
Left teat lesion	713	102	0.13	2
Right teat lesion	693	122	0.15	2
Teat lesion on either half	650	165	0.20	2

Figure 5.6: Distribution of categorical variables A) Teat Placement, B) Degree of separation and c) Udder Drop.

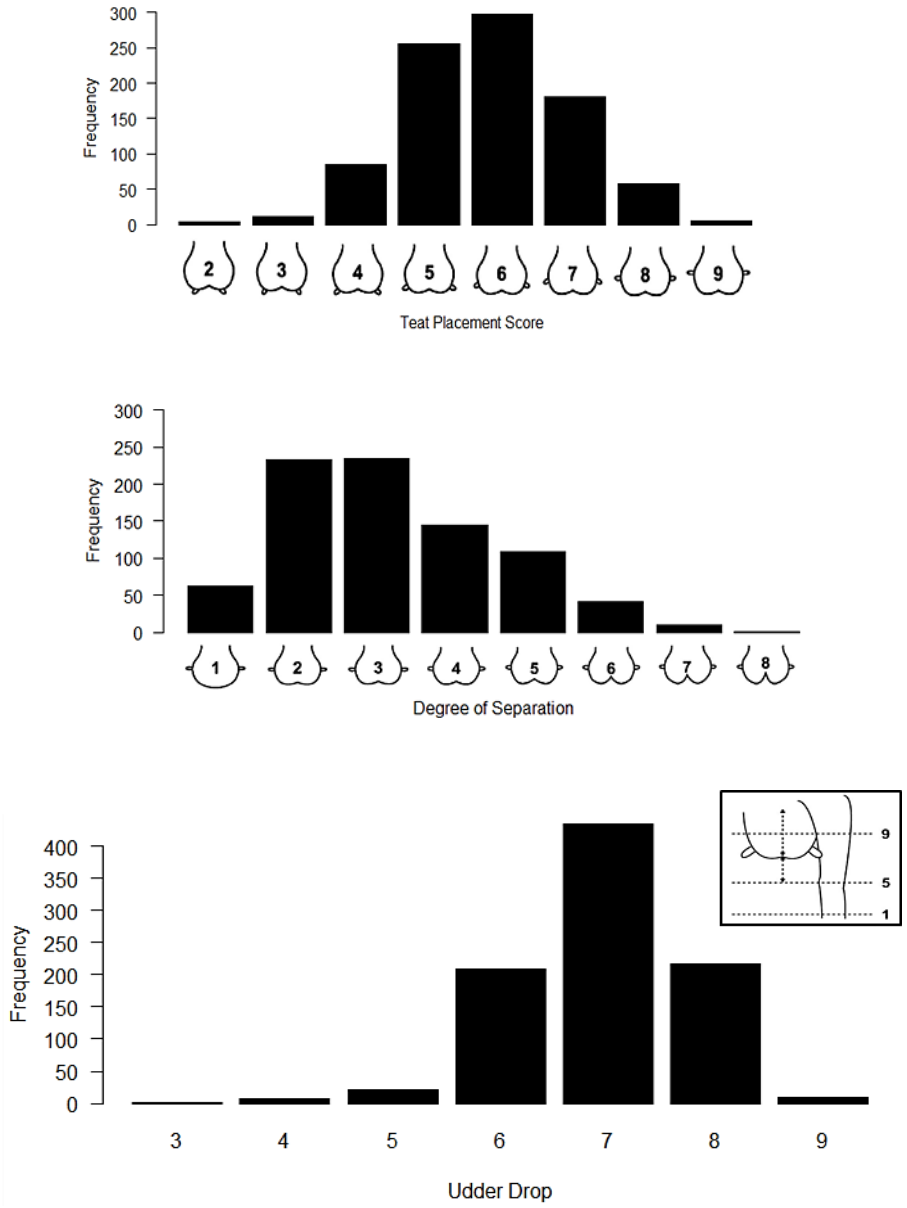
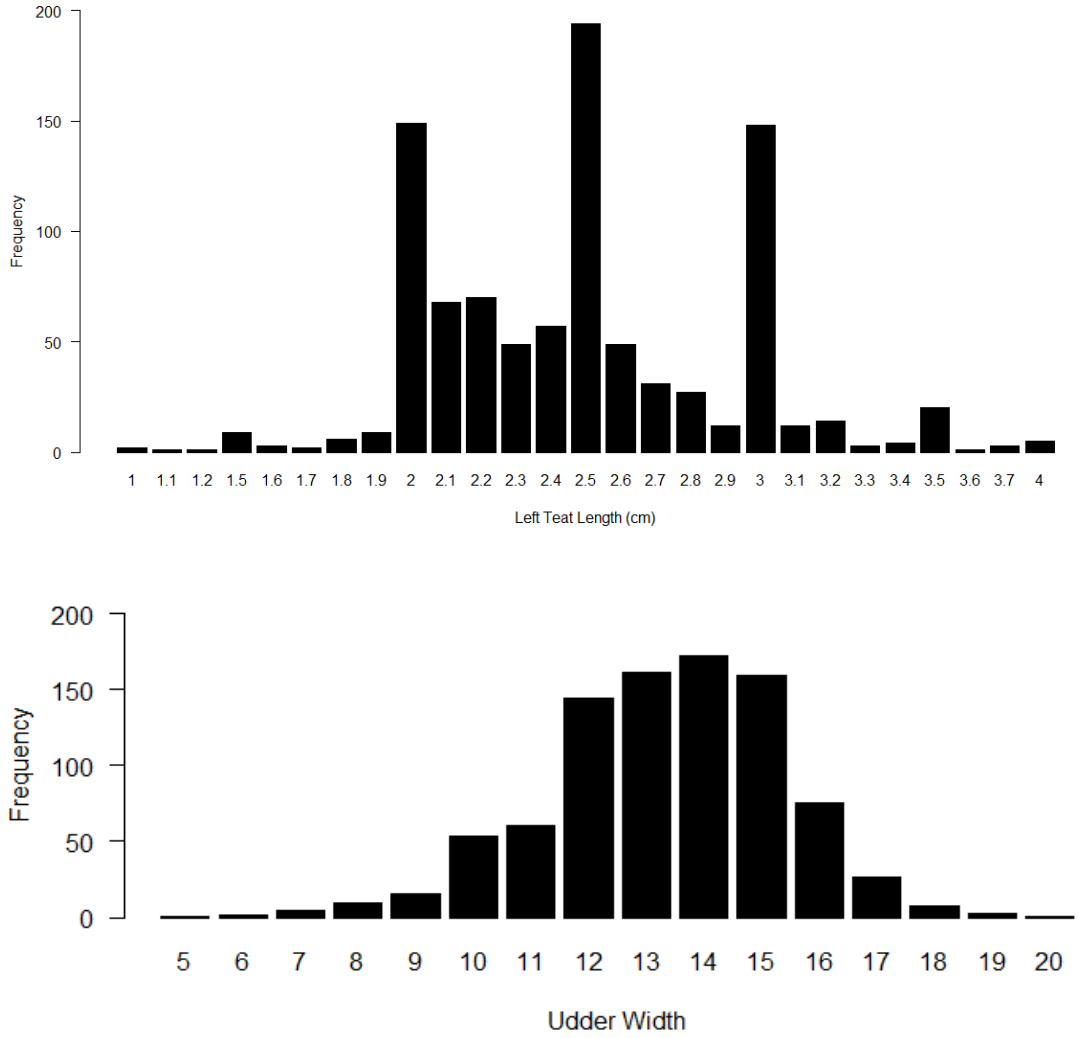


Figure 5.7: Distribution on continuous variables a) left teat length (scored to 0.5cm in year 2) and b) udder width (cm).



5.4.2. Phenotypic correlations between variables

Several of the variables were phenotypically correlated (Table 5.7). Age was significantly correlated with all udder conformation variables and body condition score, which is to be expected. Unusually, however, teat placement was correlated with both methods used for litter size calculation. Days in lactation was correlated with all udder conformation variables apart from teat placement. Body condition score was also significantly correlated with udder drop, degree of separation and left teat length. Both methods of litter size were significantly correlated, despite there being significantly fewer records in method 1.

Table 5.7: Spearman correlation values between the continuous variables. P values are given in parentheses; pairs with a p value of ≤ 0.05 are in bold.

	1	2	3	4	5	6	7	8	9
1. Age									
2. Teat Placement	**-0.066** **(0.047)**								
3. Udder Drop	**-0.268** **(0.000)**	-0.027 (0.409)							
4. Degree of Separation	**-0.075** **(0.030)**	-0.011 (0.754)	-0.062 (0.065)						
5. Udder Width	**0.075** **(0.023)**	0.042 (0.199)	**-0.432** **(0.000)**	**0.114** **(0.001)**					
6. Left Teat Length	**0.209** **(0.000)**	-0.048 (0.136)	**-0.124** **(0.000)**	**0.067** **(0.048)**	0.033 (0.314)				
7. Days in Lactation	0.031 (0.357)	0.019 (0.566)	**0.108** **(0.001)**	**0.124** **(0.000)**	**-0.291** **(0.000)**	**0.156** **(0.000)**			
8. Litter size method 1	0.012 (0.837)	**0.118** **(0.048)**	-0.280 (0.066)	0.113 (0.066)	0.096 (0.108)	0.098 (0.101)	0.012 (0.836)		
9. Litter size method 2	-0.029 (0.415)	**0.126** **(0.000)**	-0.168 (0.060)	0.069 (0.060)	**0.183** **(0.000)**	**0.123** **(0.000)**	0.057 (0.103)	**0.946** **(0.000)**	
10. Body Condition Score	**-0.164** **(0.004)**	0.074 (0.194)	**0.168** **(0.013)**	**0.145** **(0.013)**	0.006 (0.910)	**-0.117** **(0.041)**	0.025 (0.672)	-0.083 (0.160)	-0.079 (0.194)

In addition, all of the binary variables were correlated (Table 5.8).

Table 5.8: Chi-squared analyses of binary variables. P values are given in parenthesese.

	Chronic mastitis	Teat lesion left	Teat lesion right	Teat lesion any
Chronic mastitis				
Teat lesion left	**9.4 (<0.01)**			
Teat lesion right	**13.3 (<0.01)**	**169.6 (<0.01)**		
Teat lesion any	**14.2 (<0.01)**	**439.3 (<0.01)**	**531.6 (<0.01)**	

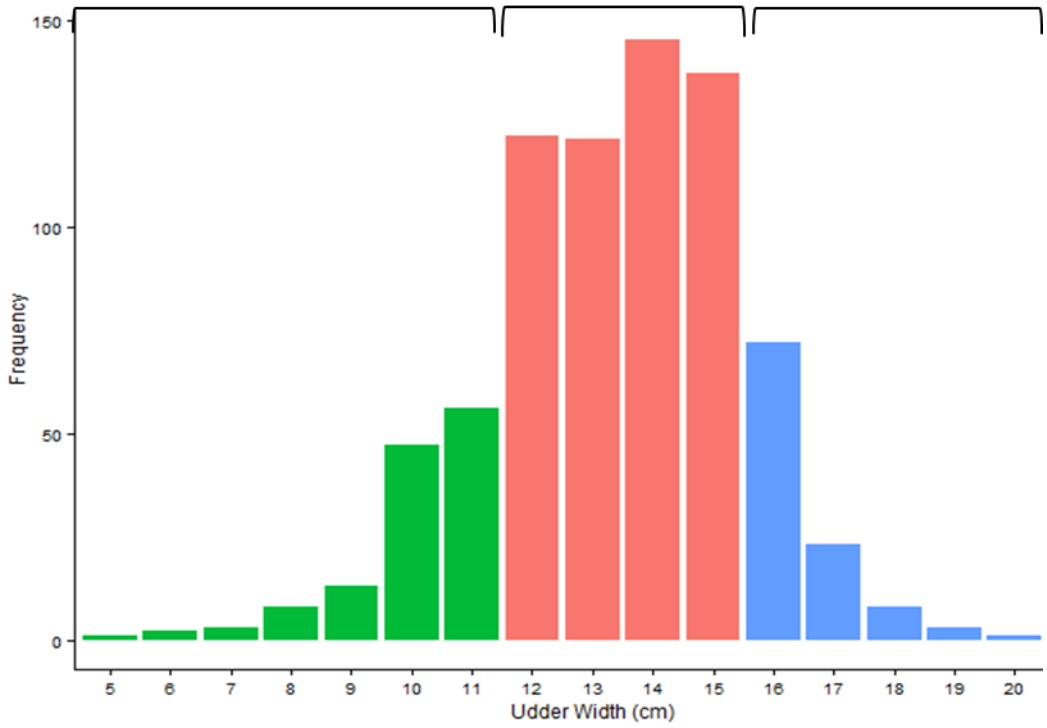
5.4.3. Comparing udder conformation traits to clinical mastitis (udder abscesses)

In order to accurately determine variables associated with whether a ewe had udder abscesses, some variables were recategorised (Table 5.9). For example udder width was categorised into narrow, medium and wide (green, pink and blue respectively in Figure 5.8).

Table 5.9: Categorisation of continuous variables for regression modelling.

Variable	Initial categories	Additional categories
Teat Placement	2,3,4,5,6,7,8,9	<6,6,>6
Udder Drop	3,4,5,6,7,8,9	<6,6,>6
Udder Width	Measurements from 5-20cm	Narrow, Medium, Wide (See Figure 8)
BCS	1-4.5 in 0.5 increments	<3,3,>3

Figure 5.8: Categorisation of the continuous variable, udder width. The variable had 3 categories: Narrow (green); Medium (Pink); and wide (blue).



Due to the correlation between udder and teat conformation traits and days in lactation and ewe age, days in lactation and ewe age were forced into the model to account for scoring at different time points in lactation for different age groups. Even after accounting for this, udder drop, udder width and the presence of a teat lesion on either teat were associated with abscesses in the udder (Table 5.10). The univariate results are in Appendix 7.

After accounting for days in lactation and ewe age, ewes with an udder drop score of >6 (higher than the hock) were less likely to have an udder abscess compared to ewes with an udder drop score of 6. As udder width increased, the risk of a ewe having an udder abscess decreased. The presence of a teat lesion on either half also increased the risk of a ewe having chronic mastitis (Table 5.10).

Table 5.10: Multivariable binomial logistic regression model for 767 observations where the outcome variable is whether the ewe had chronic mastitis.

Variable	Category	β	S.E	*P*	OR	Lower CI	Higher CI
Intercept		1.022	0.813				
Days in lactation		-0.006	0.005	0.218	0.994	0.984	1.004
Ewe age		0.099	0.571	0.082	1.104	0.015	2.223
Udder drop	<6	-0.0717	0.436	0.869	0.931	0.076	1.785
	6	Reference					
	>6	-0.687	0.210	0.001	0.503	0.091	0.915
Udder width		-0.105	0.044	0.018	0.900	0.814	0.987
Teat lesion presence	Absent	Reference					
	Present	0.522	0.202	0.010	1.685	1.289	2.081

OR- Odds Ratio, CI-Confidence Interval.

5.4.4. Udder trait heritability

The principal quantitative genetic parameter results are in Tables 5.11 and 5.12, which contain summaries of the marginal distributions of variance components and heritabilities for normally distributed and binomial traits, respectively.

The normally distributed traits are generally reasonably heritable with modes of the posterior distributions ranging from 0.10 (for Udder Width) to 0.42 (Left Teat Length), see Table 5.11. The heritability estimates of the binomial traits were lower, with the mode of sire model estimates being higher than animal model estimates for three out of four traits, see Tables 5.12 and 5.13. The modal sire model estimates of the heritability of Left and Right Teat Lesions were 0.04 and 0.14, respectively, but with large credible intervals.

Table 5.11: Summaries of marginal distributions of variance components and heritabilities from individual animal model INLA analyses of normally distributed traits.

Trait	Parameter	Mean	sd	Percentiles			Mode
				2.5%	50%	97.5%	
Teat Placement	σ_e^2	0.67	0.08	0.53	0.67	0.82	0.67
	σ_a^2	0.36	0.09	0.20	0.35	0.55	0.34
	h^2	0.35	0.04	0.27	0.35	0.43	0.35
Udder Drop	σ_e^2	0.50	0.05	0.40	0.50	0.59	0.50
	σ_a^2	0.13	0.05	0.04	0.12	0.24	0.11
	h^2	0.21	0.04	0.13	0.21	0.30	0.21
Degree of Separation	σ_e^2	1.32	0.15	1.05	1.31	1.60	1.32
	σ_a^2	0.47	0.16	0.21	0.45	0.81	0.42
	h^2	0.27	0.04	0.19	0.27	0.36	0.27
Udder Width	σ_e^2	2.61	0.20	2.22	2.61	3.00	2.62
	σ_a^2	0.36	0.18	0.09	0.33	0.79	0.25
	h^2	0.12	0.04	0.05	0.11	0.20	0.10
Left Teat Length	σ_e^2	0.11	0.01	0.08	0.11	0.14	0.11
	σ_a^2	0.08	0.02	0.05	0.08	0.12	0.08
	h^2	0.42	0.04	0.35	0.42	0.50	0.42

Table 5.12: Summaries of marginal distributions of heritabilities from individual animal model INLA analyses of binomial traits.

Trait	Parameter	Mean	sd	Percentiles			Mode
				2.5%	50%	97.5%	
Chronic Mastitis	σ_a^2	0.33	0.22	0.03	0.29	0.85	0.18
	h^2	0.09	0.05	0.01	0.08	0.20	0.06
Left Teat Lesion	σ_a^2	0.41	0.30	0.03	0.33	1.07	0.12
	h^2	0.10	0.07	0.01	0.09	0.25	0.04
Right Teat Lesion	σ_a^2	0.44	0.34	0.03	0.36	1.31	0.16
	h^2	0.11	0.07	0.01	0.10	0.28	0.06
Any Teat Lesion	σ_a^2	0.33	0.24	0.02	0.27	0.88	0.11
	h^2	0.09	0.06	0.01	0.07	0.21	0.04

Table 5.13: Summaries of marginal distributions of heritabilities from sire model INLA analyses of binomial traits.

Trait	Parameter	Mean	sd	Percentiles			Mode
				2.5%	50%	97.5%	
Chronic Mastitis	σ_s^2	0.16	0.11	0.02	0.14	0.43	0.10
	σ_a^2	0.65	0.44	0.07	0.56	1.71	0.39
	h^2	0.18	0.12	0.02	0.16	0.46	0.12
Left Teat Lesion	σ_s^2	0.12	0.08	0.01	0.11	0.29	0.03
	σ_a^2	0.49	0.33	0.03	0.44	1.16	0.13
	h^2	0.14	0.09	0.01	0.13	0.32	0.04
Right Teat Lesion	σ_s^2	0.23	0.17	0.02	0.20	0.67	0.11
	σ_a^2	0.94	0.69	0.07	0.79	2.70	0.45
	h^2	0.26	0.17	0.02	0.23	0.68	0.14
Any Teat Lesion	σ_s^2	0.15	0.10	0.01	0.13	0.35	0.04
	σ_a^2	0.60	0.41	0.04	0.51	1.42	0.16
	h^2	0.17	0.11	0.01	0.15	0.39	0.05

Fixed effect models

Table 5.14 contains the deviance information criteria from individual animal model analysis of various combinations of the primary fixed effects. The inclusion of flock by scoring date (FSD) effects was enforced, and combinations of ewe age (EA) and days in milk (DIM) were included alongside it. The combination of fixed effects corresponding to the lowest value of DIC for each trait was used in all other analyses reported here.

Table 5.14: Deviance information criteria (DIC) for individual animal models with various combinations of fixed effects included. The lowest (best) value of DIC for each trait is in bold type.

Trait	Included fixed effects			
	FSD	FSD, EA	FSD, DIM	FSD, EA, DIM
Teat Placement	2022.61	2019.92	2022.49	**2019.37**
Udder Drop	1718.41	**1708.25**	1719.69	1708.94
Degree of Separation	**2290.07**	2291.51	2291.11	2292.41
Udder Width	2907.05	2894.59	2899.37	**2884.11**
Left Teat Length	725.71	725.93	**719.12**	722.22
Chronic Mastitis	**902.48**	904.66	904.30	906.46
Left Teat Lesion	**531.30**	533.24	531.97	533.87
Right Teat Lesion	**572.57**	573.20	574.07	574.71
Any Teat Lesion	**699.28**	702.33	701.04	704.08

Inclusion of repeated records

Tables 5.15 and 5.16 contain summaries of posterior distributions of variance components from analyses including repeated records. Only for Udder Drop, Degree of Separation and Udder Width was the estimate of the permanent environmental variance sufficient to make an estimate of the repeatability noticeably greater than the heritability.

Table 5.15: Summaries of marginal posterior distributions of variance components, heritabilities, and repeatabilities of continuous traits estimates with an individual animal model and including repeated records.

Trait	Parameter	Mean	s.d.	Percentiles			Mode
				2.5%	50%	97.5%	
Teat Placement	σ_e^2	0.6340	0.0634	0.5180	0.6320	0.7600	0.6280
	σ_a^2	0.4220	0.0819	0.2760	0.4180	0.5880	0.4110
	σ_{pe}^2	0.0003	0.0015	0.0000	0.0001	0.0017	0.0000
	h^2	0.4010	0.0369	0.3300	0.4010	0.4740	0.4010
	r	0.4010	0.0369	0.3300	0.4010	0.4750	0.4020
Udder Drop	σ_e^2	0.3070	0.0415	0.2300	0.3050	0.3870	0.2980
	σ_a^2	0.1560	0.0532	0.0664	0.1530	0.2670	0.1500
	σ_{pe}^2	0.1660	0.0544	0.0798	0.1620	0.2750	0.1510
	h^2	0.2440	0.0439	0.1620	0.2430	0.5970	0.5170
	r	0.5170	0.0418	0.4340	0.5180	0.5970	0.5170
Degree of Separation	σ_e^2	1.0400	0.1200	0.8100	1.0400	1.2700	1.0400
	σ_a^2	0.5260	0.1500	0.2510	0.5190	0.8280	0.5030
	σ_{pe}^2	0.2010	0.1410	0.0461	0.1600	0.5260	0.0447
	h^2	0.2720	0.0443	0.1890	0.2700	0.3620	0.2680
	r	0.4200	0.0465	0.3290	0.4200	0.5100	0.4240

Trait	Parameter	Mean	s.d.	Percentiles			Mode
				2.5%	50%	97.5%	
Udder Width	σ_e^2	2.3200	0.2110	1.8800	2.3300	2.6900	2.3600
	σ_a^2	0.4220	0.1790	0.1440	0.3970	0.8230	0.2780
	σ_{pe}^2	0.1870	0.1940	0.0295	0.0968	0.6980	0.0294
	h^2	0.1290	0.0394	0.0621	0.1260	0.2140	0.1150
	r	0.2340	0.0494	0.1410	0.2320	0.3340	0.2390
Left Teat Length	σ_e^2	0.0992	0.0111	0.0793	0.0988	0.1210	0.0980
	σ_a^2	0.0850	0.0153	0.0577	0.0843	0.1160	0.0833
	σ_{pe}^2	0.0002	0.0005	0.0000	0.0001	0.0015	0.0000
	h^2	0.4620	0.0360	0.3910	0.4620	0.5330	0.4720
	r	0.4630	0.0360	0.3920	0.5330	0.4720	0.4630

Table 5.16: Summaries of marginal posterior distributions of variance components of binary traits estimated with an individual animal model and including repeated records.

Trait	Parameter	Mean	s.d.	Percentiles			Mode
				2.5%	50%	97.5%	
Chronic mastitis	σ_a^2	0.36278	0.23669	0.04078	0.31901	0.92031	0.21607
	σ_{pe}^2	0.00050	0.00242	0.00001	0.00007	0.00174	0.00002
	h^2	0.09666	0.05219	0.02226	0.08840	0.21917	0.05832
	r	0.09671	0.05219	0.02230	0.08844	0.21919	0.05925
Left Teat Lesion	σ_a^2	0.48234	0.35379	0.04008	0.40653	1.39118	0.19756
	σ_{pe}^2	0.00038	0.00165	0.00001	0.00007	0.00166	0.00002
	h^2	0.12342	0.07273	0.02300	0.11073	0.29431	0.06695
	r	0.12346	0.07273	0.02306	0.11076	0.29435	0.06692
Right Teat Lesion	σ_a^2	0.56167	0.38060	0.05980	0.48630	1.48308	0.33203
	σ_{pe}^2	0.00035	0.00152	0.00001	0.00007	0.00162	0.00002
	h^2	0.14128	0.07426	0.03278	0.13040	0.31263	0.10039
	r	0.14132	0.07425	0.03283	0.13045	0.31267	0.10042
Any Teat Lesion	σ_a^2	0.37653	0.26795	0.03245	0.32027	1.06284	0.16672
	σ_{pe}^2	0.00044	0.00203	0.00001	0.00007	0.00167	0.00002
	h^2	0.09919	0.05885	0.01841	0.08897	0.23909	0.05199
	r	0.09923	0.05884	0.01846	0.08900	0.23918	0.06561

Inclusion of litter size

Litter size was included in analysis of single ewe record data using an animal model for normally distributed traits and a sire model for the binomial traits. The results of these analyses are summarised in Tables 5.17 and 5.18.

Comparing the modes of the posterior distributions of heritability estimates for normally distributed and binary traits with and without litter size included (Tables 5.17 and 5.18), the modal heritability was generally slightly lower when litter size was included in the model. It was only higher for Teat Placement and Chronic Mastitis.

Table 5.17: Summaries of marginal distributions of variance components and heritabilities from individual animal model INLA analyses of normally distributed traits when litter size was included in or excluded from the model.

Trait	Litter size	Parameter	Mean	sd	Percentiles			Mode
					2.5%	50%	97.5%	
Teat Placement	Yes	σ_e^2	0.714	0.078	0.571	0.712	0.868	0.710
		σ_a^2	0.302	0.089	0.150	0.296	0.487	0.284
		h^2	0.303	0.042	0.223	0.302	0.387	0.303
	No	σ_e^2	0.713	0.078	0.571	0.711	0.867	0.710
		σ_a^2	0.302	0.089	0.150	0.296	0.486	0.284
		h^2	0.303	0.042	0.224	0.303	0.387	0.299
Udder Drop	Yes	σ_e^2	0.494	0.048	0.403	0.494	0.586	0.497
		σ_a^2	0.108	0.051	0.029	0.102	0.218	0.084
		h^2	0.187	0.044	0.108	0.185	0.278	0.189
	No	σ_e^2	0.496	0.050	0.403	0.496	0.591	0.499
		σ_a^2	0.122	0.053	0.039	0.117	0.237	0.100
		h^2	0.206	0.044	0.126	0.204	0.297	0.199
Degree of Separation	Yes	σ_e^2	1.292	0.150	1.020	1.290	1.583	1.295
		σ_a^2	0.504	0.171	0.228	0.490	0.862	0.454
		h^2	0.288	0.044	0.205	0.288	0.377	0.284
	No	σ_e^2	1.285	0.150	1.014	1.283	1.575	1.286
		σ_a^2	0.513	0.171	0.235	0.499	0.870	0.465
		h^2	0.293	0.044	0.209	0.292	0.381	0.294

Trait	Litter size	Parameter	Mean	sd	Percentiles			Mode
					2.5%	50%	97.5%	
Udder Width	Yes	σ_e^2	2.533	0.193	2.152	2.535	2.910	2.544
		σ_a^2	0.323	0.172	0.085	0.292	0.732	0.213
		h^2	0.106	0.038	0.043	0.103	0.191	0.097
	No	σ_e^2	2.583	0.204	2.187	2.584	2.982	2.594
		σ_a^2	0.368	0.189	0.094	0.336	0.810	0.260
		h^2	0.116	0.039	0.051	0.113	0.203	0.106
Left Teat Length	Yes	σ_e^2	0.111	0.014	0.085	0.111	0.139	0.111
		σ_a^2	0.076	0.018	0.046	0.075	0.113	0.072
		h^2	0.405	0.040	0.328	0.405	0.484	0.405
	No	σ_e^2	0.110	0.014	0.084	0.110	0.138	0.110
		σ_a^2	0.080	0.018	0.049	0.079	0.117	0.077
		h^2	0.421	0.039	0.344	0.421	0.498	0.423

Table 5.18: Summaries of marginal distributions of sire and additive genetic variances and heritabilities from sire model INLA analyses of binomial traits when litter size was included in or excluded from the model.

Trait	Litter size	Parameter	Mean	sd	Percentiles			Mode
					2.5%	50%	97.5%	
Chronic Mastitis	Yes	σ_s^2	0.158	0.109	0.015	0.136	0.428	0.090
		σ_a^2	0.632	0.435	0.061	0.544	1.712	0.359
		h^2	0.180	0.117	0.019	0.159	0.460	0.113
	No	σ_s^2	0.148	0.105	0.013	0.126	0.416	0.079
		σ_a^2	0.593	0.420	0.054	0.504	1.664	0.315
		h^2	0.169	0.113	0.016	0.148	0.449	0.100
Left Teat Lesion	Yes	σ_s^2	0.114	0.075	0.007	0.102	0.267	0.030
		σ_a^2	0.454	0.300	0.030	0.407	1.067	0.121
		h^2	0.132	0.084	0.009	0.120	0.300	0.038
	No	σ_s^2	0.114	0.075	0.008	0.105	0.265	0.031
		σ_a^2	0.458	0.298	0.030	0.418	1.059	0.123
		h^2	0.133	0.084	0.009	0.123	0.298	0.040
Right Teat Lesion	Yes	σ_s^2	0.221	0.165	0.016	0.182	0.636	0.098
		σ_a^2	0.882	0.660	0.065	0.728	2.543	0.392
		h^2	0.243	0.168	0.020	0.210	0.648	0.127
	No	σ_s^2	0.229	0.170	0.018	0.191	0.661	0.108
		σ_a^2	0.918	0.679	0.071	0.764	2.645	0.432
		h^2	0.253	0.171	0.022	0.220	0.669	0.139
Any Teat Lesion	Yes	σ_s^2	0.113	0.075	0.007	0.100	0.266	0.030
		σ_a^2	0.452	0.300	0.030	0.401	1.065	0.120
		h^2	0.131	0.084	0.009	0.118	0.299	0.038
	No	σ_s^2	0.130	0.086	0.008	0.114	0.304	0.034
		σ_a^2	0.514	0.345	0.034	0.455	1.216	0.138
		h^2	0.149	0.096	0.010	0.134	0.338	0.044

Sensitivity to priors

Figures 5.9 and 5.10 summarise the results of the analyses with varying priors for normally distributed and binomial traits, respectively. The graphs show the mode and 95% confidence interval of the posterior distribution of the heritability for each trait under each set of priors.

Where observed data provide little information, it would be expected that the posterior distribution would resemble the prior distribution: the modes and would be similar and the credible interval would reflect the shape of the prior distribution. From the figures it can be seen that where the priors were informative (low 95% cut-off point for the prior distribution of the heritability), the posterior mode was closer to the mode of the prior distribution than where the prior was less informative. For the normally distributed traits (Figure 5.9), the credible interval of the heritability was reasonably constant across priors. For the binomial traits (Figure 5.10) the 95% credible interval varies with the width of the prior distribution.

Our priors for this study were derived from a prior distribution of the heritability with a mode of 0.15 and a 95% cut-off point at 0.7. For both the normally distributed and binary traits this does not appear to have unduly influenced the results; with the mode and 95% credible intervals for our chosen prior being similar to those obtained when a uniform prior for the heritability was used.

Figure 5.9: Mode and 95% confidence interval of posterior marginal distribution of heritability of continuous traits as the mode and informativeness of the prior distribution of heritability varies.

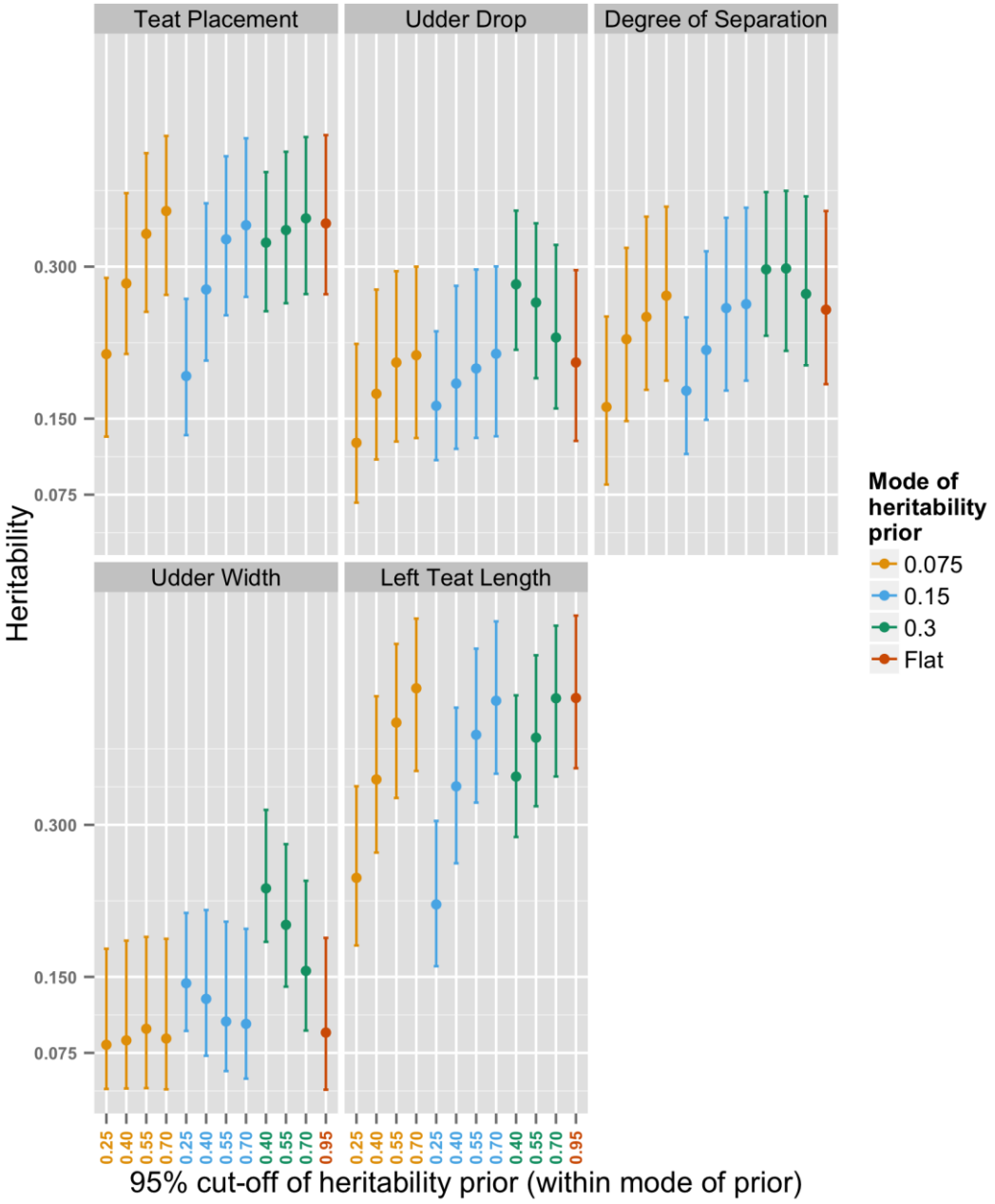
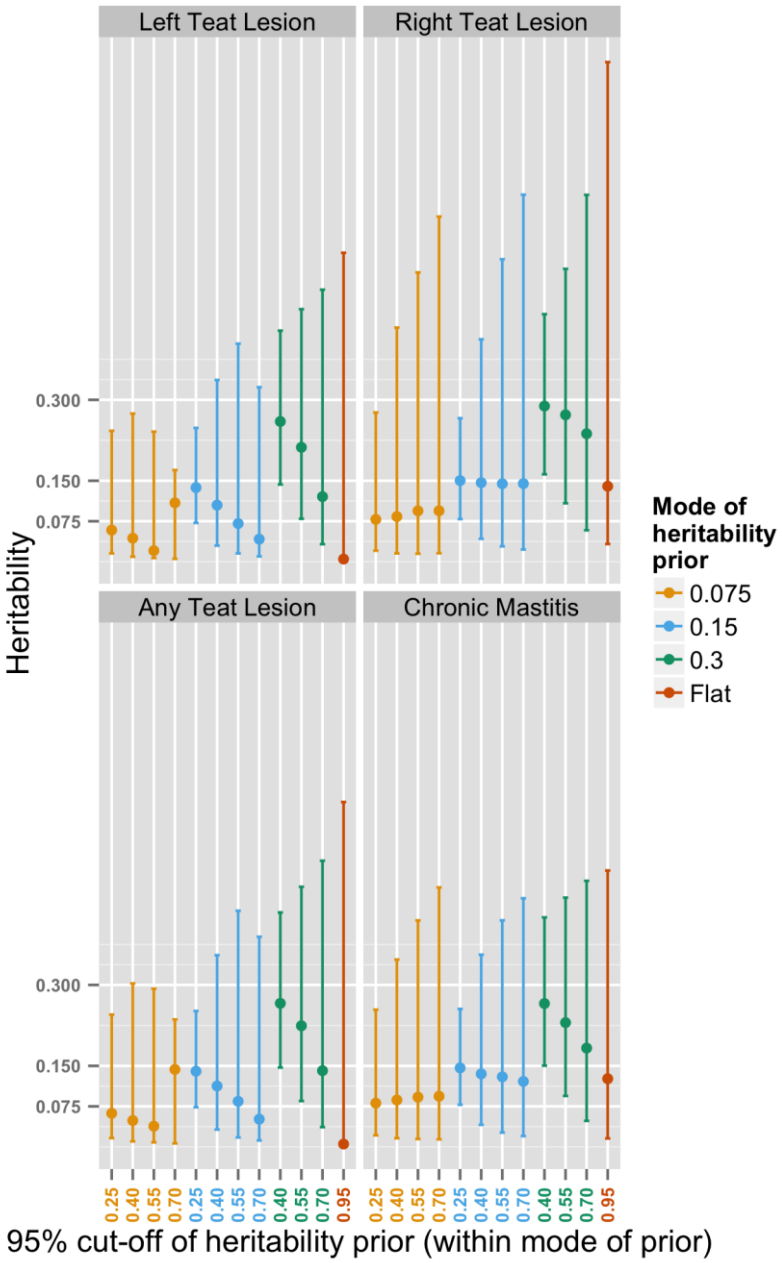


Figure 5.10: Mode and 95% confidence interval of posterior marginal distribution of heritability of binary traits as the mode and informativeness of the prior distribution of heritability varies.



5.5. Discussion

This analysis initially included 968 observations for 873 ewes. After removing duplicate values and ewes without pedigree data for the genetic modelling, a total of 817 records for 740 ewes remained in the quantitative genetic analysis of udder trait data of Texel ewes.

All udder traits bar teat placement were significantly associated with at least one other trait, which is to be expected. However the reason for the lack of association between teat placement and other udder conformation traits is not clear.

In this study, ewe age was phenotypically correlated with all udder conformation traits, indicating the importance of accounting for it in statistical analyses. The number of days into lactation at which ewes were scored was also correlated with all udder conformation traits bar teat placement, indicating the other traits may be linked to milk yield and/or udder volume. This has been shown previously, where udder depth and length were correlated to milk production (Pérez-Cabal *et al.* 2013). This is further supported by the significant correlation of body condition score with these traits found in this study.

All udder conformation traits but degree of separation were significantly associated with chronic mastitis in the univariable analyses. In the final model, only udder drop and udder width were included. This is likely to be because of the association of these variables with other udder conformation traits- resulting in them being dropped from the model. This supports previous studies where udder conformation traits have been associated with somatic cell count ewes (Huntley *et al.* 2012, Pérez-Cabal *et al.* 2013).

The normally distributed traits generally show good levels of heritability, with only Udder Width (h^2 = 0.10) having a h^2 less than 0.2. This would indicate that selection on any of the normally distributed traits should be feasible given sufficient data recording. The heritabilities in this study are higher than those found in previous studies which ranged from 0.06 and 0.26 (de la Fuente *et al.* 2011, Fernández *et al.* 1997, Serrano *et al.* 2002) . Each of these studies had much larger numbers of ewes (3977, 2015 and 7523 ewes respectively). In addition these analyses were based on

dairy Churra or Manchega ewes which could explain the similar heritabilties found in these studies, and the difference between these studies and ours.

Heritabilities of binary traits were slightly higher under a sire model, with the posterior distributions more skewed towards higher values but the modes similar to those from animal model analyses. Simulation and previous analyses in the literature would indicate that the sire model estimates may be better estimates of the true h^2.

The binomial traits had estimated heritabilities on the underlying scale of between 4% and 14%. The levels of heritability for Chronic Mastitis and Right Teat Lesions would imply that selection could be feasible in these traits, given sufficient recording to ensure reasonable levels of accuracy of selection (many related animals recorded). The difference of 0.10 between the modal estimates of the heritability of Left and Right Teat Lesions, when they would be expected to be highly similar traits, may indicate the influence of random sampling in our small data set. This would show the danger of over-interpreting the results here, in particular for binary traits where each observation is less informative than an observation on a normally distributed trait.

Non-zero estimates of the permanent environmental variance were only observed for Udder Drop, Degree of Separation and Udder Width. Given the data structure, permanent environmental variances may be detected for other traits when there are considerably more ewes with repeated observations. The binary nature of some traits makes estimation of this effect more problematic.

Inclusion of litter size in the model has some small impact upon the heritability estimates of the normally distributed traits. This is consistent with these traits being genetically correlated with litter size such that the genetic variance includes some variation due to litter size.

These analyses have provided estimates of the heritabilities of udder and teat conformation traits and Chronic Mastitis in Texel ewes. For inclusion of any of these traits in a selection programme further information would be required. Without knowledge of the genetic correlations among these traits and with other traits, it is not possible to predict the genetic progress that would be made in these traits or the correlated genetic progress in other traits. In particular, there is no information on the genetic relationship between these traits and the tendency of ewes to suffer from mastitis.

Chapter 6. General Discussion

The aim of this thesis was to contribute to the understanding of the development and persistence of mastitis in suckler ewes. The likelihood of a ewe developing mastitis is influenced by a farmer's management practices, the environment of the ewe, the ewe's susceptibility to developing mastitis and the bacterial strains in the environment and mammary gland. Due to the multifactorial nature of these infections, several research approaches were used in this thesis.

The initial study estimated an incidence rate of clinical mastitis (IRCM) of 1.2 ewes/100 ewes/year, the first estimate that indicates the impact of mastitis on suckler flocks in England. The estimate varied depending on management system, with ewes that always reared lambs outdoors having a lower IRCM than those that always reared lambs indoors. In addition, the risk for an increase in IRCM varied by management system and temporally. This indicated that different management systems expose ewes to different risks and therefore need to be treated independently when considering risk factors for the development of mastitis. Whilst the IRCM obtained is the same as the only other IRCM in suckler ewes (Arsenault *et al.* 2008), there were some respondents that never checked their ewes for mastitis. Only 88% of respondents checked ewes' mammary glands for disease at lambing and the regularity of checks decreased over time after lambing (Chapter 2), therefore this IRCM may be an underestimate.

In addition, ewe movement was significantly associated with IRCM: flocks that were kept in the same barns during and after lambing, or in the same field before and after lambing had a decreased IRCM. A likely explanation for this is that when ewes are moved they are exposed to new potential pathogens and so suffer more mastitis. The potential importance of the microbial environment was also highlighted by the association between floor type and IRCM, which is likely to be linked to the how easy it is for a flooring material to be cleaned and/or the effect of flooring type on how saturated bedding becomes. Hard flooring, associated with a decrease in IRCM, is uneven which could improve fluid drainage and so impede bacterial colonisation. In order to ascertain this link, this hypothesis requires further investigation.

Finally, poor udder conformation was significantly positively correlated with IRCM. This questionnaire-based study helped generate the hypothesis that udder conformation is significantly associated with chronic mastitis This hypothesis was subsequently tested in Chapter 5, where udder conformation traits were found to be significantly associated with chronic mastitis. This highlights the usefulness of questionnaire-based studies for hypothesis generation and subsequent testing in observational studies.

The role of the environment was first considered by investigating the potential sources of mastitis-causing pathogens (Chapter 3). In order to effectively link an environmental organism to a case of mastitis, it is necessary to identify organisms to at least the strain level. The current gold standard strain typing methods (MLST, genotyping and PFGE) can be extremely time-consuming and expensive, particularly for epidemiological studies where samples need to be taken from multiple ewes over time. MALDI-ToF-MS offered a quicker, cheaper alternative to these methods and therefore was compared to PFGE in order to investigate whether this method could be used as a strain typing tool.

The results from the study showed that MALDI-ToF-MS had good correspondence with PFGE when utilising composite correlation indices (CCI) for *Staphylococcus warneri, Staphylococcus equorum, Rhodococcus corprophilus, Bacillus pumilus* and *Micrococcus luteus* to varying levels – most species had distinct strain level cut-off points for CCI estimates, whereas the threshold for *Staphylococcus warneri* was more ambiguous.

For example, MALDI-ToF-MS analysis, supported by PFGE, indicated that many different strain types of *Staphylococcus equorum* were isolated from different samples. These data suggest there are many different sources of *Staphylococcus equorum* which fits the traditional view of this organism having an “environmental” origin. The “environmental” versus “contagious” classifications, although rigid, does provide useful information as to potential transmission pathways of bacterial species in mastitis.

The same species and strain types for *Rhodococcus corprophilus,* and *Staphylococcus warneri* were identified on udder skin and in ewe milk, indicating

the udder skin as a possible reservoir of mastitis causing pathogens. However as the study in Chapter 3 was not a longitudinal study, the direction of transmission (if any) could not be ascertained.

In Chapter 3 only blood agar was used to culture bacteria from udder skin and milk samples which could have limited the variety of bacterial species isolated and then identified. In addition, isolates were prepared for MALDI-ToF-MS using the direct smear method which results in increased spectral variability and signal to noise ratio and therefore greater technical CCI variability. The study design was improved in a larger, longitudinal study (Chapter 4) with varied media and sampling which provided the ability to investigate potential transmission pathways. As udder skin was identified as a potential reservoir of pathogenic bacteria (Chapter 3), this chapter focussed on potential transmission pathways via lamb mouths and udder skin by sampling these environments and ewe milk over time.

To maximise the success rate of finding potential transmission pathways of mastitis causing bacteria, a variety of selective media were used in the study. In addition, a formic acid extraction method was used instead of the direct smear method to reduce spectral variability.

Despite only sampling from ewes showing no clinical signs, a variety of mastitis-causing pathogens were isolated from ewe milk, udder skin and lamb mouths. This differs to chapter 3 where only a few mastitis-causing pathogens were identified despite ewes showing clinical signs of mastitis. This could be due to the increased number of samples collected in chapter 4. Alternatively, the use of selective media in this study may have enhanced the growth of these species, highlighting the importance of media selection to ensure optimal conditions for bacterial isolation.

Twenty-six species were found in more than one location (lamb mouth, ewe udder skin and/or ewe milk). The fact that some bacterial species were found in all 3 locations and some in only 2 of 3 locations may give some indication as to their transmission pathways. For example, species only present in lamb mouths and milk may be directly transmitted between lamb and ewe. Alternatively, isolates only identified on ewe skin and in the milk may be transferred from the ewe's environment to the skin and then the milk rather than via her lamb.

To the authors knowledge, *Mannheimia haemolytica* is the only mastitis-causing pathogen isolated from lamb mouths previously (Fragkou *et al.* 2011). In this study, a variety of bacteria associated with mastitis were isolated from lamb mouths, including: *Aerococcus viridans, Bacillus licheniformis, Escherichia coli, Enterococcus faecalis, Enterococcus faecium* and coagulase-negative staphylococci, providing evidence for the role of lambs in the transmission of mastitis.

Potential transmission events over time were observed between lamb mouths, ewe udder skin and milk. Most notably, the same *Escherichia coli* strain type was isolated from lamb mouths and udder skin at the first observation after lambing but not on udder skin pre-lambing or in ewe milk.

In addition, the same strain types of several coagulase-negative staphylococci were isolated at different sampling time points in ewe milk and on udder skin, including initial detection on udder skin followed by subsequent detection in the milk at the next observation. This represents evidence of transmission from the udder skin to the milk of a potential pathogen. There were also several cases of colonisation of the same strain type on udder skin and in ewe milk at the same observation. Whilst these data suggest passage between udder skin and ewe milk it is not possible to determine the direction of transmission.

The persistent isolation of the same strain types were found over time for *Bacillis licheniformis, Staphylococcus hominis,* and *Staphylococcus warneri* for ewe milk and *Staphylococcus equorum* and *Staphylococcus aureus* on ewe udder skin. Due to the long intervals between sampling points, these may represent re-colonisations by the same strain rather than persistence. However, these persistent isolations occurred in a large number of ewes, therefore it seems reasonable to assume these were persistent colonisations, particularly in the case of ewe milk.

These results indicate the capacity of MALDI-ToF-MS as a rapid strain identification method to determine potential transmission events and persistence of bacterial strains between and within lamb mouths, ewe udder skin and/or ewe milk over time. The identification of these potential transmission pathways are particularly important as they highlight potential routes for infection for other

mastitis causing pathogens as well and so may inform general treatment and control strategies for mastitis.

Having investigated farmer management practices and the environment of the ewe in the role of mastitis, the ewe's susceptibility to developing mastitis also needed to be investigated. In particular, having identified udder conformation as the most significant factor associated with IRCM in Chapter 2, the final study aimed to investigate whether udder and teat conformation are heritable traits that affect mastitis.

In this study (Chapter 5), all udder traits (bar teat placement) were associated with the number of days in lactation, indicating that these traits were correlated to milk production. Ewe age was also correlated with all udder traits. It was therefore important to account for ewe age and days in lactation in the association between udder traits and chronic mastitis. Even after accounting for these factors, there were phenotypic association between udder traits and chronic mastitis. This supports the hypothesis generated in Chapter 2, where the IRCM was positively associated with the percent of flock with poor udder conformation and highlights the importance of this factor. Interestingly, the presence of a teat lesion on either half also increased the risk of a ewe having chronic mastitis. Although this was a tested factor in chapter 2, it was not significantly associated with IRCM. In this study (Chapter 5) researchers did thorough checks on all the mammary glands on ewes in the study whilst the questionnaire based study in Chapter 2 indicated that many farmers only check their animals irregularly. As teat lesions are fairly transient in nature they may well have been missed with irregular checking, which may explain the discrepancy between these two studies.

The udder conformation traits showed good levels of heritability for all but udder width, and for chronic mastitis and teat lesions on the right udder half indicating that selection on these traits could be feasible, provided there was sufficient data recording. Although these analyses provided good estimated of the heritabilities of udder traits, knowledge of genetic correlations between these traits and others is required to determine the genetic progress that would be made if these traits were included in a selection program.

The work in this thesis has addressed several gaps in the knowledge in mastitis epidemiology as outlined in Chapter 1. Namely by providing the first estimate for the IRCM in suckler flocks in England, and generating hypotheses for factors that may affect the IRCM. One of the associated factors, udder conformation, was investigated in a cohort study as to whether these traits are heritable and whether they influence chronic mastitis cases. Finally, the role of the ewe's environment was investigated by identifying the potential sources and transmission pathways for a variety of mastitis causing bacteria. This thesis highlights the value of a multidisciplinary approach utilising microbiology and epidemiology whereby a cohort of ewes could be investigated over time using microbiological techniques in order to improve our understanding of the development of mastitis.

In conclusion, the results from these studies have contributed to our understanding of influences on the development of mastitis and generated hypotheses for future studies to reduce IRCM in suckler flocks.

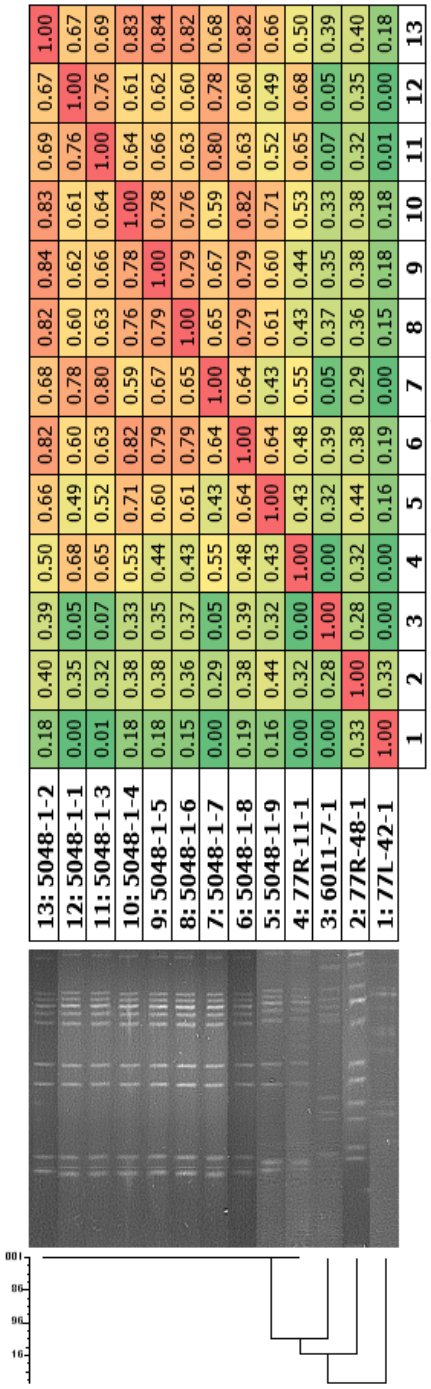
6.1. Further work

Further work could include testing the hypotheses linked to the ewe's microbial environment generated in Chapter 1, in particular the effect of flooring type on the IRCM and whether this is linked to bedding saturation and therefor microbial colonisation. The effect of ewe movement between environments on the IRCM was another interesting hypothesis that could be tested further by investigating bacterial communities in different environments for example individual lambing pens, group lambing pens and each field.

Further evidence for the potential transmission pathways highlighted within this thesis could be gathered using shorter sampling periods using single nucleotide polymorphisms (SNP). In addition, the somatic cell count could be compared to this dataset in order to observe whether somatic cell count (indicating an immune response) increases after a potential transmission event.

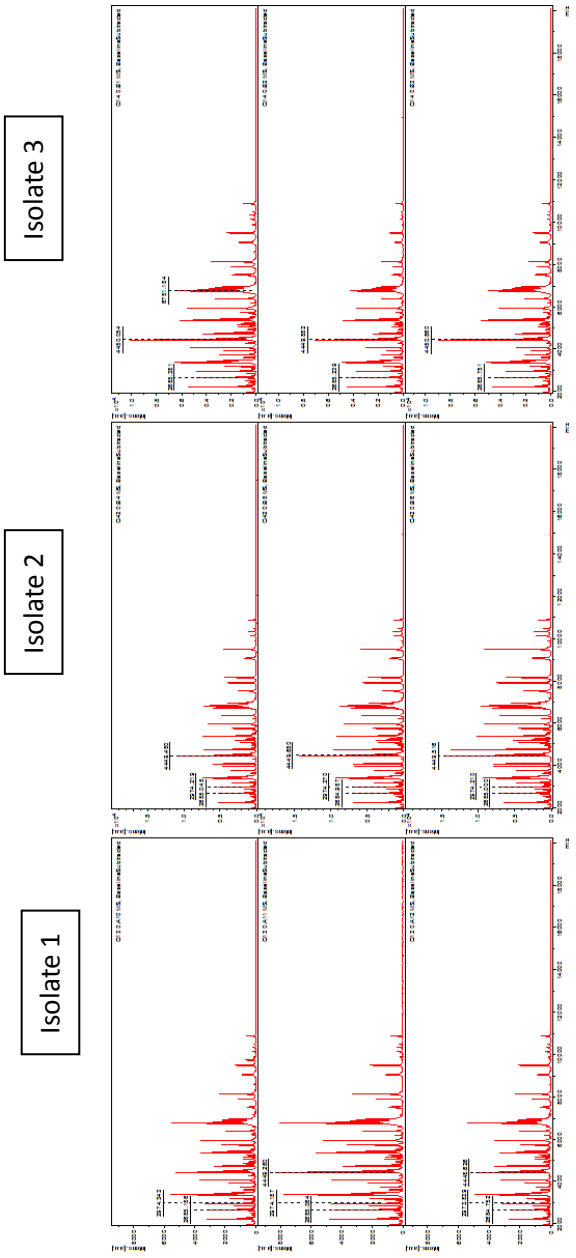
Appendices

Appendix 1: Comparison of pulsed-field gel electrophoresis gels, derived dendrograms, and colour coded composite correlation indices for *Staphylococcus warneri* (green represents lowest estimates, and red highest).



	1	2	3	4	5	6	7	8	9	10	11	12	13
13: 5048-1-2	0.18	0.40	0.39	0.50	0.66	0.82	0.68	0.82	0.84	0.83	0.69	0.67	1.00
12: 5048-1-1	0.00	0.35	0.05	0.68	0.49	0.60	0.78	0.60	0.62	0.61	0.76	1.00	0.67
11: 5048-1-3	0.01	0.32	0.07	0.65	0.52	0.63	0.80	0.63	0.66	0.64	1.00	0.76	0.69
10: 5048-1-4	0.18	0.38	0.33	0.53	0.71	0.82	0.59	0.76	0.78	1.00	0.64	0.61	0.83
9: 5048-1-5	0.18	0.38	0.35	0.44	0.60	0.79	0.67	0.79	1.00	0.78	0.66	0.62	0.84
8: 5048-1-6	0.15	0.36	0.37	0.43	0.61	0.79	0.65	1.00	0.79	0.76	0.63	0.60	0.82
7: 5048-1-7	0.00	0.29	0.05	0.55	0.43	0.64	1.00	0.65	0.67	0.59	0.80	0.78	0.68
6: 5048-1-8	0.19	0.38	0.39	0.48	0.64	1.00	0.64	0.79	0.79	0.82	0.63	0.60	0.82
5: 5048-1-9	0.16	0.44	0.32	0.43	1.00	0.64	0.43	0.61	0.60	0.71	0.52	0.49	0.66
4: 77R-11-1	0.00	0.32	0.00	1.00	0.43	0.48	0.55	0.43	0.44	0.53	0.65	0.68	0.50
3: 6011-7-1	0.00	0.28	1.00	0.00	0.32	0.39	0.05	0.37	0.35	0.33	0.07	0.05	0.39
2: 77R-48-1	0.33	1.00	0.28	0.32	0.44	0.38	0.29	0.36	0.38	0.38	0.32	0.35	0.40
1: 77L-42-1	1.00	0.33	0.00	0.00	0.16	0.19	0.00	0.15	0.18	0.18	0.01	0.00	0.18

Appendix 2: technical replicates for 3 *Streptococcus uberis* isolates



Appendix 3: *Staphylococcus equorum* analytes isolated from ewe W40 pre and post-lambing over multiple observations

Record date	Visit	Organism	Milk/Skin	Analyte
Approx 1 week pre-lambing	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S MC D*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S MC B*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S MC A*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S BD C*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S MC C*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S BD B*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S BD A*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S BD D*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S MC C*
	1	*Staphylococcus equorum*	Skin Pre-lamb	*W40 S MC A*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43380 S BD B*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43793 S BD B1*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43380 S MC A*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43793 S BD B2*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43793 S BD B*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43380 S MC D*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43380 S MC C*
24-Mar-11	2	*Staphylococcus equorum*	Skin	*43793 S MC B*
21-Apr-11	3	*Staphylococcus equorum*	Skin	*44141 S MC C*
21-Apr-11	3	*Staphylococcus equorum*	Skin	*44141 S MC B*
21-Apr-11	3	*Staphylococcus equorum*	Skin	*44141 S MC A*
21-Apr-11	3	*Staphylococcus equorum*	Skin	*44141 S MC F*
19-May-11	4	*Staphylococcus equorum*	Skin	*44752 S MC B*
19-May-11	4	*Staphylococcus equorum*	Skin	*44752 S MC D*
19-May-11	4	*Staphylococcus equorum*	Skin	*44752 S MC A*

Appendix 4: Corresponding composite correlation indices estimates for *Staphylococcus equorum* isolates retrieved pre and post lambing skin samples for ewe W40

22: W40 S MC D																					1.00
21: W40 S MC C																				1.00	0.91
20: W40 S MC B																			1.00	0.80	0.86
19: W40 S MC A																		1.00	0.85	0.72	0.78
18: W40 S BD C																	1.00	0.81	0.90	0.84	0.90
17: W40 S BD B																1.00	0.94	0.84	0.93	0.84	0.90
16: W40 S MC D															1.00	0.39	0.39	0.39	0.40	0.45	0.44
15: W40 S MC C														1.00	0.38	0.24	0.25	0.24	0.23	0.40	0.37
14: W40 S MC B													1.00	0.93	0.41	0.28	0.28	0.27	0.27	0.43	0.39
13: W40 S MC A												1.00	0.87	0.89	0.51	0.34	0.36	0.34	0.35	0.52	0.48
12: W40 S BD D											1.00	0.91	0.90	0.93	0.49	0.29	0.30	0.30	0.30	0.46	0.41
11: W40 S BD B										1.00	0.92	0.87	0.88	0.92	0.39	0.22	0.23	0.22	0.23	0.40	0.35
10: W40 S BD A									1.00	0.92	0.91	0.89	0.90	0.91	0.44	0.27	0.28	0.26	0.27	0.44	0.39
9: 44141 S MC F								1.00	0.77	0.73	0.72	0.68	0.70	0.72	0.44	0.20	0.21	0.19	0.20	0.35	0.31
8: 44141 S MC C							1.00	0.86	0.66	0.58	0.63	0.58	0.61	0.58	0.43	0.20	0.20	0.20	0.20	0.34	0.28
7: 44141 S MC B						1.00	0.75	0.88	0.77	0.77	0.75	0.76	0.82	0.85	0.40	0.21	0.22	0.20	0.21	0.33	0.30
6: 44141 S MC A					1.00	0.63	0.66	0.69	0.61	0.62	0.60	0.55	0.64	0.61	0.38	0.18	0.05	0.01	0.18	0.29	0.08
5: 43793 S MC B				1.00	0.92	0.01	0.24	0.25	0.22	0.20	0.16	0.21	0.27	0.21	0.19	0.42	0.92	0.89	0.81	0.90	0.77	0.83
4: 43793 S BD B1			1.00	0.95	0.95	0.23	0.26	0.28	0.26	0.19	0.16	0.20	0.27	0.17	0.17	0.40	0.90	0.88	0.76	0.86	0.79	0.83
3: 43380 S MC D		1.00	0.96	0.95	0.94	0.21	0.23	0.27	0.25	0.19	0.16	0.20	0.27	0.18	0.17	0.44	0.87	0.85	0.75	0.84	0.77	0.80
2: 43380 S MC C	1.00	0.92	0.90	0.93	0.87	0.22	0.26	0.29	0.26	0.26	0.22	0.29	0.33	0.27	0.23	0.48	0.86	0.83	0.79	0.84	0.76	0.79
1: 43380 S BD B	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22

Appendix 5: Composite correlation indices for isolates retrieved from milk samples over time for *Bacillus licheniformis* for ewe W40

6: 21-Apr-11 (Visit 2) *44141 MC A*						1.0
5: 24-Mar-11 (Visit 1) *43793 MC D*					1.0	0.4
4: 24-Mar-11 (Visit 1) *43793 MC D1*				1.0	0.4	0.4
3: 24-Mar-11 (Visit 1) *43793 MC B*			1.0	0.7	0.0	0.3
2: 24-Mar-11 (Visit 1) *43793 MC C*		1.0	0.4	0.6	0.4	0.8
1: 24-Mar-11 (Visit 1) *43588 MC B*	1.0	0.6	0.3	0.4	0.0	0.6
	1	2	3	4	5	6

Appendix 6: Composite correlation indices for isolates retrieved from milk samples over time for *Staphylococcus hominis* for ewe OR15

4: 9-May-11 (Visit 3) *44462 BD A1*				1.00
3: 9-May-11 (Visit 3) *44462 BD A*			1.00	0.64
2: 11-Apr-11 (Visit 2) *44387 MC A1*		1.00	0.72	0.90
1: 11-Apr-11 (Visit 2) *44387 MC A*	1.00	0.49	0.53	0.52
	1	2	3	4

Appendix 7: Univariate binomial logistic regression where the outcome variable is whether the ewe had chronic mastitis.

Variables		N	%	B	SE	*P*	OR	Lower CI	Higher CI
Age (categorical)	1	24	5.6	-2.265	1.044	0.030	0.104	-1.942	2.150
	2	268	26.6	-0.422	0.224	0.059	0.656	0.218	1.094
	3	191	30.9	-0.222	0.235	0.345	0.801	0.340	1.262
	4	116	33.9	-0.125	0.265	0.637	0.882	0.363	1.402
	5+ years	145	35.9	Reference			1.0		
Teat Placement	2	3	100.0	15.448	509.7	0.976	$5.12x10^6$	$5.12x10^6$	$5.12x10^6$
	3	11	54.5	1.065	0.622	0.087	2.900	1.682	4.118
	4	68	39.7	0.489	0.286	0.087	1.631	1.071	2.191
	5	191	29.3	0.017	0.212	0.935	1.018	0.602	1.433
	6	248	29.0	Reference			1.0		
	7	178	27.0	-0.104	0.221	0.639	0.901	0.468	1.335
	8	59	37.3	0.401	0.311	0.198	1.493	0.883	2.102
	9	5	40.0	0.477	0.924	0.606	1.611	-0.199	3.421
Teat Placement (categorised)	<5	82	43.9	0.642	0.275	0.019	1.900	1.362	2.438
	5	490	29.4	Reference			1.0		
	>5	191	29.3	0.001	0.188	0.997	1.001	0.632	1.369
Udder drop	3	2	50.0	0.338	1.422	0.812	1.403	-1.385	4.190
	4	7	14.3	-1.453	1.090	0.183	0.234	-1.903	2.371
	5	22	50.0	0.243	0.462	0.599	1.275	0.370	2.180
	6	187	41.7	Reference			1.0		
	7	333	28.2	-0.574	0.193	0.003	0.563	0.186	0.941
	8	201	23.9	-0.794	0.223	0.000	0.452	0.014	0.889
	9	11	27.3	-0.355	0.723	0.623	0.701	-0.715	2.118
Udder drop (categorised)	<6	31	41.9	-0.067	0.401	0.867	0.935	0.148	1.722
	6	545	26.6	Reference			1.0		
	>6	187	41.7	-0.649	0.178	0.000	0.523	0.174	0.872
Degree of separation	1	52	32.7	0.262	0.345	0.447	1.300	0.624	1.976
	2	195	26.2	-0.054	0.228	0.812	0.947	0.501	1.394
	3	199	27.6	Reference			1.0		
	4	125	22.4	-0.256	0.267	0.339	0.774	0.250	1.299
	5	93	22.6	-0.277	0.294	0.347	0.758	0.181	1.335
	6	37	29.7	0.135	0.395	0.733	1.144	0.370	1.918
	7	10	10.0	-1.242	1.066	0.244	0.289	-1.800	2.378
	8	1	100.0	14.522	535.4	0.978	$2.03x10^6$	$2.03x10^6$	$2.03x10^6$
Udder width	Narrow	130	36.9	0.358	0.208	0.084	1.431	1.024	1.838
	Medium	525	28.4	Reference			1.0		
	Wide	107	35.5	0.306	0.224	0.172	1.358	0.919	1.798

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